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USE OF MYCOBACTERIAL VACCINES IN CD4$^+$ OR CD8$^+$ LYMPHOCYTE-DEFICIENT MAMMALS

Statement Regarding Federally Sponsored Research or Development

The U.S. Government has a paid-up license in this invention and the right in limited circumstances to require the patent owner to licence others on reasonable terms as provided by the terms of AI26170 awarded by National Institutes of Health.

Background of the Invention

(1) Field of the Invention

The present invention generally relates to live bacterial vaccines. More specifically, the invention is related to novel *Mycobacterium sp.* compositions, and the use of those compositions to protect mammals against disease caused by virulent *Mycobacterium sp.*

(2) Description of the Related Art

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There exists an urgent need for a novel tuberculosis (TB) vaccine as there are more than 8 million new cases of tuberculosis and more than 2 million deaths reported each year by the WHO (Dye et al., 1999). The discovery of the causative agent of TB, *Mycobacterium tuberculosis*, by Robert Koch in 1882 opened up the possibility for a novel vaccine (Koch, 1882). Since then, numerous attempts to develop attenuated vaccines against tuberculosis have

failed, including tuberculin (a protein extract of killed tubercle bacilli) developed by Dr. Koch himself. This failure of tuberculin to protect led to a "firm conviction that immunity could only be established by inducing a definite, albeit limited, tuberculosis process" Grange et al., 1983). Thus, numerous labs set out to follow the example of Dr. Louis Pasteur for viruses and enrich attenuated mutants of the tubercle bacillus following repeated passaging.

In order to test the hypothesis that a tubercle bacillus isolated from cattle (now known as *M. bovis*) could transmit pulmonary tuberculosis following oral administration, Drs. Calmette and Guerin developed a medium containing beef bile that enabled the preparation of fine homogenous bacillary suspensions (Calmette and Guerin, 1905). An *M. bovis* strain obtained from Dr. Norcard, was passaged every 21 days in this medium and after the 39^{th} passage, the strain was found to be unable to kill experimental animal (Gheorghiu, 1996). "Between 1908 and 1921, the strain showed no reversion to virulence after 230 passages on bile potato medium" (Id.), which is consistent with the attenuating mutation being a deletion mutation. In the animal studies that followed, the strain ('BCG') was found to be attenuated but it also protected animals receiving a lethal challenge of virulent tubercle bacilli (Calmette and Guerin, 1920). BCG was first used as a vaccine against tuberculosis in 1921. From 1921 to 1927, BCG was shown to have protective efficacy against TB in a study on children (Weill-Halle and Turpin, 1925; Calmette and Plotz, 1929) and adopted by the League of Nations in 1928 for widespread use in the prevention of tuberculosis. By the 1950's after a series of clinical trials, the WHO was encouraging widespread use of BCG vaccine throughout the world (Fine and Rodrigues, 1990). Although an estimated 3 billion doses have been used to vaccinate the human population against tuberculosis, the mechanism that causes BCG's attenuation remains unknown.

Mahairas et al. (1996) first compared the genomic sequences of BCG and *M. bovis* using subtractive hybridization and found that there were three major deletions (named RD1, RD2, and RD3) present in the genome of *M. bovis*, but missing in BCG. Behr et al. (1999) and others (Gordon et al., 2001) later identified 16 large deletions, including RD1 to RD3, present in the BCG genome but absent in *M. tuberculosis*. These authors concluded that 11 of these 16 deletions were unique to *M. bovis*, while the remaining 5 deletions were unique to BCG. They also found that one of these 5 deletions, designated RD1 (9454 bp), is present in all of the BCG substrains currently used as TB vaccines worldwide and concluded that the deletion of RD1 appeared to have occurred very early during the development of BCG, probably prior to 1921 (Behr et al., 1999).

The development of insertional mutagenesis systems for BCG and *M. tuberculosis* (Kalpana et al., 1991), transposon mutagenesis systems (Cirillo et al., 1991; McAdam et al., 1995; Bardarov et al., 1997) and allelic exchange systems (Balasubramanian et al., 1996; Pelicic et al., 1997) led to the isolation of the first auxotrophic (nutrient-requiring) mutants of these slow-growing mycobacteria. Auxotrophic mutants of BCG and *M. tuberculosis* have been shown to confer protection to *M. tuberculosis* challenges with variable efficacies (Guleria et al., 1996; Smith et al., 2001). However, a head-to-head comparison of BCG to a leucine auxotroph of BCG showed that a single immunization elicited no positive skin-test and imparted little immunity to challenges with *M. tuberculosis* or *M. bovis* (Chambers et al., 2000). In contrast, a methionine auxotroph of BCG that grows *in vivo* did confer significant protection to challenge to both *M. tuberculosis* and *M. bovis* (Id.). A single dose of a leucine auxotroph of *M. tuberculosis* failed to elicit protection as good as BCG in BALB/c mice (Hondalus et al., 2000). These results suggest that optimal immunity against *M. tuberculosis* requires some growth of the immunizing strain. Double mutants of *M. tuberculosis* have also been created (Parish and Stoker, 2000), but whether such mutants are improved over single attenuating mutants in protecting mammals against challenge with a virulent mycobacterium, particularly when the host is immunocompromised, has not been established.

It is also worth noting that in the study of Chambers *et al.* (2000), both BCG and the BCG mutants seemed to protect better against *M. bovis* challenge than *M. tuberculosis*. If we assume the reverse correlate is true, we could hypothesize that optimal immunity against *M. tuberculosis* could be achieved with *M. tuberculosis*-derived mutant that grew in the mammalian host.

Based on the above, there remains a need for improved live mycobacterial vaccines having attenuated virulence, that confer protection from virulent mycobacteria, particularly *M. tuberculosis*. The need is particularly acute for immunocompromised individuals. The instant invention satisfies that need.

Summary of the Invention

The present invention is based on the discovery that mycobacteria having two attenuating mutations are safe and protect mammals that are deficient in $CD4^+$ and/or $CD8^+$ lymphocytes from challenge by virulent mycobacteria.

Thus, the present invention is directed to methods of treating a mammal that does not have severe combined immune deficiency but is deficient in $CD4^+$ lymphocytes. The methods

comprise inoculating the mammal with an attenuated mycobacterium in the *Mycobacterium tuberculosis* (*M. tuberculosis*) complex. In these embodiments, the mycobacterium comprises two deletions, where a virulent mycobacterium in the *M. tuberculosis* complex having either deletion exhibits attenuated virulence.

The invention is also directed to methods of treating a mammal that does not have severe combined immune deficiency but is deficient in $CD8^+$ lymphocytes. The methods comprise inoculating the mammal with an attenuated mycobacterium in the *Mycobacterium tuberculosis* (*M. tuberculosis*) complex. In these embodiments, the mycobacterium comprises two deletions, where a virulent mycobacterium in the *M. tuberculosis* complex having either deletion exhibits attenuated virulence.

Additionally, the invention is directed to the use of an attenuated mycobacterium in the *Mycobacterium tuberculosis* (*M. tuberculosis*) complex for the manufacture of a medicament for treatment of a mammal that is that does not have severe combined immune deficiency but is deficient in $CD4^+$ lymphocytes. In these embodiments, the mycobacterium comprises two deletions, where a virulent mycobacterium in the *M. tuberculosis* complex having either deletion exhibits attenuated virulence.

In other embodiments, the invention is directed to the use of an attenuated mycobacterium in the *Mycobacterium tuberculosis* (*M. tuberculosis*) complex for the manufacture of a medicament for treatment of a mammal that is that does not have severe combined immune deficiency but is deficient in $CD8^+$ lymphocytes. In these embodiments, the mycobacterium comprises two deletions, where a virulent mycobacterium in the *M. tuberculosis* complex having either deletion exhibits attenuated virulence.

Brief Description of the Drawings

FIG. 1 shows maps and autoradiographs pertaining to the construction of Δ*RD1* mutants of *M. tuberculosis*. Panel a, *M. tuberculosis* H37Rv published sequence between 4346 kb and 4364 kb, showing predicted *NcoI* sites. Arrows on the top represent the genes in the RD1 region. The RD1 region deleted from *M. bovis* BCG is represented by an open bar spanning from *Rv3871* to *Rv3879c*. Upstream and downstream flanking sequences, UFS and DFS respectively, are indicated as closed bars underneath the grid line. Panel b, Southern hybridization of *M. tuberculosis* H37Rv Δ*RD1* created using two-step sequential homologous recombination. Panel c, Southern hybridization of *M. tuberculosis* H37Rv and Erdman ΔRD1 strains created using specialized transduction.

FIG. 2 shows graphs summarizing experiments establishing that *M. tuberculosis* H37Rv Δ*RD1* is attenuated in SCID mice. Panel a, Seven female SCID mice were infected intravenously with $2X10^6$ CFU *M. tuberculosis* H37Rv, *M. tuberculosis* H37Rv Δ*RD1*, and *M. tuberculosis* H37Rv Δ*RD1*::2F9 per mouse. The number of surviving mice was recorded post infection. Panel b, Mice were infected with different doses of *M. tuberculosis* H37Rv, *M. tuberculosis* H37Rv Δ*RD1*, and *M. bovis* BCG. For each strain, infection doses of $2X10^6$ CFU, $2X10^5$ CFU, $2X10^4$ CFU, and $2X10^3$ CFU per mouse, were administered via tail intravenous injection.

FIG. 3 is photographs, micrographs and autoradiographs showing that the *M. tuberculosis* H37Rv Δ*RD1* mutant exhibits two distinct colonial morphotypes. Panel a, *M. tuberculosis* H37Rv. Panel b, *M. tuberculosis* H37Rv Δ*RD1*. Panel c, *M. tuberculosis* H37Rv Δ*RD1*::2F9. Panel d, Southern analysis of *M. tuberculosis* H37Rv Δ*RD1* *Nco*I-digested genomic DNA, isolated from three smooth and three rough colonies and probed with DFS. Panels e–g, Colonial morphotypes at higher magnification. e, Smooth morphotype at week 4. f, Rough morphotype at week 4. g, Rough morphotype at week 6.

FIG. 4 is graphs showing the growth kinetics of *M. tuberculosis* H37Rv Δ*RD1* in BALB/c mice. Mice were infected with $2X10^6$ CFU through tail injection. Time to death was noted and at day 1, week 4, 8, 14, and 22 post-infection, mice were sacrificed to determine the mycobacterial burden in the spleen, liver, and lung. The numbers represent the means of CFUs in organs derived from three animals. The error bars represent the standard errors of the means. Panel a, Time to death assay in BALB/c mice. Panel b, Spleen. Panel c, Liver. Panel d, Lung.

FIG. 5 is micrographs from pathological studies of infected BALB/c mice. Panels a – c, Lungs from mice infected with $2x10^6$ CFU of *M. tuberculosis* H37Rv examined at 4, 8 and 14 weeks post-infection. The mild to moderate pneumonia at 4 and 8 weeks (a and b) progressed to severe consolidating granulomatous pneumonia at 14 weeks post infection (c). Panels d – f, Lungs from mice infected with $2x10^6$ CFU of *M. tuberculosis* H37Rv Δ*RD1* examined at 4, 8 and 22 weeks post-infection showing moderate pneumonia at 8 weeks post-infection (e) and persistent bronchitis and multifocal pneumonitis at 22 weeks post-infection (f). Panels (g)– (i), Mild lung lesions from mice infected with $2x10^6$ CFU of BCG at 4, 8 and 22 weeks post-infection. Mild focal granulomas scattered widely in the lung at each time point with predominately lymphocytic accumulations in foci at 22 weeks post-infection.

FIG. 6 shows graphs summarizing experiments establishing that pantothenate auxotrophy leads to attenuation of *M. tuberculosis* Δ*panCD* mutant in SCID mice. Panel A, Survival of BALB/c SCID mice (n = 12 per group) infected intravenously with 490 CFU of H37Rv (○) or 210 CFU of *panCD* complementing strain (*panCD* in single copy integrated into the chromosome)(●) or 3.4 x 10^5 CFU of Δ*panCD* mutant (▲) or 3.3 x 10^5 CFU of BCG-P (□). Panel B, Bacterial numbers in the spleen (●) and lungs (▲) of SCID mice infected intravenously with 490 CFU of H37Rv or numbers in spleen (○) and lungs (Δ) of mice infected with 3.4 x 10^5 CFU of Δ*panCD* mutant. The results represent means ± standard errors of four to five mice per group.

FIG. 7 shows graphs summarizing experiments demonstrating the attenuation, limited growth and persistence of Δ*panCD* mutant in immunocompetent mice. Panel A, Survival of BALB/c mice (n=12 per group) infected with 4.4 x 10^6 CFU of wild-type *M. tuberculosis* H37Rv (○), 3.2 x 10^6 CFU *panCD* complementing strain (*panCD* in single copy integrated into the chromosome)(●) or 2.4 x 10^6 CFU *panCD* mutant (▲). Panels B and C, Bacterial loads in spleen and lungs of BALB/c mice infected intravenously with 4.4 x 10^6 CFU wild-type H37Rv (o) or 3.2 x 10^6 CFU *panCD* complementing strain(●) or 2.4 x 10^6 CFU Δ*panCD* mutant (▲). CFUs were assayed at various time points on 7H11 agar with or without pantothenate supplementation where required. The results represent means ± standard errors of four to five mice per group.

FIG. 8 shows graphs summarizing experiments demonstrating the attenuation, limited replication and persistence of Δ*nadBC* mutant in immunocompetent mice. Panels A and B, Bacterial loads in lungs and spleen of C57BL/6 mice infected with wild type *M. tuberculosis* H37Rv (●) or Δ*nadBC* mutant (○). Mice were infected intravenously with 10^6 CFU of each strain. CFUs were assayed at various time points on 7H11 agar with or without nicotinamide supplementation where required. The results represent means ± standard errors of four to five mice per group. Panel C, Survival of C57BL/6 mice (n=12 per group) infected with 10^6 CFU of wild-type bacteria (○) or 10^6 CFU of Δ*nadBC* mutant (○).

FIG. 9 shows an illustration, map and autoradiograph relating to the pathway for the biosynthesis of pantothenic acid and coenzyme A and its disruption in *M. tuberculosis*. Panel a. The enzymes involved in the biosynthesis of pantothenic acid and having annotation in the genomic sequence of *M. tuberculosis* H37Rv are shown in bold numbers: 1) panB, ketopantoate hydroxymethyl transferase; 2) panD, aspartate-1-decarboxylase; 3) panC, pantothenate synthetase; 4) panK, pantothenate kinase; 5) acpS, ACP synthase. Panel b. Map

of the panCD genomic region in the wild type M. tuberculosis H37Rv and the Δ*panCD* mutant. Restriction sites and probe location are indicated. Panel c. Southern blot of *BssH*II-digested genomic DNA from wild-type H37Rv (lane 1), two independent clones of Δ*panCD* mutant from H37Rv (lanes 2 & 3) and probed with a 716 bp downstream region flanking the panCD operon. Molecular size marker (in kb) is shown on the left.

FIG. 10 shows graphs summarizing experiments demonstrating that pantothenate auxotrophy leads to attenuation of Δ*panCD* mutant in mice. a. Survival of BALB/c SCID mice (n = 12 per group) infected intravenously with H37Rv (○) or panCD-complemented strain (●) or Δ*panCD* mutant (▲) or M. bovis BCG-P (□). b. Bacterial numbers in the spleen (○), liver (□) and lung (Δ) of SCID mice infected intravenously with H37Rv or the bacterial numbers in the spleen (◍), liver (▦) and lung (◭) of mice infected with Δ*panCD* mutant. c. Survival of immunocompetent BALB/c mice (n = 16 per group) infected with H37Rv (○) or panCD-complemented strain (◍) or Δ*panCD* mutant (◭). d, e, f. Bacterial numbers in lung (d), spleen (e) and liver (f) of immunocompetent BALB/c mice infected intravenously with either H37Rv (○), *panCD*-complemented strain (●) or Δ*panCD* mutant (▲). Data are means ± standard errors of four to five mice per group.

FIG. 11 shows micrographs and graphs summarizing experiments demonstrating that the Δ*panCD* mutant produces less tissue pathology in lungs of infected BALB/c mice and protects mice against challenge with virulent *M. tuberculosis*. a. Severe consolidating granulomatous pneumonia (★) obliterating the normal lung parenchyma at 3 weeks post-infection with H37Rv. b. Severe consolidating granulomatous pneumonia (★) obliterating the normal lung parenchyma at 3 weeks post-infection with the *panCD*-complemented strain, similar to the wild type strain. c. Mild lung infection caused by the Δ*panCD* mutant at 3 weeks post-infection. Localized multifocal granulomas (arrows) scattered widely in the lung. Most of the lung is normal alveolar spaces and airways. d. Lung of mouse infected with Δ*panCD* mutant examined histologically at 23 weeks post-infection. Occasional focal, mild perivascular and interstitial infiltrations composed of predominately lymphocytes (arrows). Most of the lung is normal alveolar spaces and airways. e, f. The attenuated Δ*panCD* mutant protects mice against aerogenic challenge with virulent *M. tuberculosis* Erdman. Subcutaneously immunized mice were challenged after 90 days through the aerosol route. The CFU numbers reflect the bacterial burden at 28 days post aerosol challenge in the lung (e) and spleen (f). Naive mice - black fill; mice infected with 1 dose

panCD - light shade; mice infected with 2 doses *panCD* - dark shade; mice infected with BCG-P - unshaded.

FIG. 12 shows autoradiographs of Southern analysis of the NcoI-digested genomic DNA isolated from the wild type and the Δ*RD1* mutants generated using specialized transduction in *M. tuberculosis* and *M. bovis*. Lanes: 1 - *M. tuberculosis* H37Rv; 2 - *M. tuberculosis* H37Rv Δ*RD1*; 3 - *M. tuberculosis* Erdman; 4 - *M. tuberculosis* Erdman Δ*RD1*; 5 - *M. tuberculosis* CDC1551; 6 - *M. tuberculosis* CDC1551 Δ*RD1*; 7 - *M. bovis* Ravenel; and 8 - *M. bovis* Ravenel Δ*RD1*. The probed used in the Southern analysis was either DFS (left) or IS6110-specific sequence (right).

FIG. 13 shows graphs summarizing data confirming that deletion of *RD1* in *M. tuberculosis* and *M. bovis* confers an attenuation of virulence for *M. tuberculosis* and *M. bovis*, as indicated by these Time to death curves of mice infected intravenously with 2x10^6 CFU mycobacteria. Panel A, SCID mice infected with *M. tuberculosis* H37Rv (▩), *M. tuberculosis* H37Rv Δ*RD1* (□), *M. tuberculosis* Erdman (◉), *M. tuberculosis* Erdman Δ*RD1* (○), *M. tuberculosis* CDC1551 (▲), *M. tuberculosis* CDC1551 Δ*RD1* (Δ), *M. bovis* Ravenel (▼), *M. bovis* Ravenel Δ*RD1* (∇); Panel B, SCID mice infected intravenously with *M. tuberculosis* H37Rv (●), *M. tuberculosis* Δ*RD1* (■), *M. tuberculosis* Δ*RD1*::2F9 (▲), *M. bovis* Ravenel (○), *M. bovis* Ravenel Δ*RD1* (□), and *M. bovis* BCG (Δ); Panel C, BALB/c mice were infected with *M. tuberculosis* H37Rv (○), *M. tuberculosis* Δ*RD1* (Δ), and *M. bovis* BCG (□).

FIG. 14 is graphs summarizing experiments demonstrating the clearance of the lysine auxotroph in SCID mice. The viable bacterial counts are shown for the spleens, livers, and lungs of SCID mice injected intravenously with the lysine auxotroph strain and the prototrophic control strain. Three mice were assayed at each time point. The error bars indicate the standard deviations of the mean values. Note that the counts at time zero are the counts obtained at 24 hours post-injection, as described in Example 5. Panels A, B and C show the log of the viable bacteria in each organ after injection with 1x10^7 CFU of the *Lys$^-$ M. tuberculosis* mutant mc^{2}3026 (□), or 1x10^7 CFU of the complemented *Lys$^+$ M. tuberculosis* strain mc^{2}3026/pYUB651 (□).

FIG. 15 is graphs summarizing experimental results of experiments that establish the vaccine efficacy of the *M. tuberculosis* lysine auxotroph mc^{2}3026. C57Bl/6 mice were injected intravenously with 1x10^6 CFU of the *M. tuberculosis* lysine auxotroph mc^{2}3026, followed by one or two additional injections at 4 week intervals. Five mice were sacrificed weekly after each immunization and the viable bacteria counts of the auxotroph determined in

the lungs and spleens. Control mice were given a similar amount of BCG-Pasteur or only PBST. Shown in Panel A is the clearance of the auxotroph from the lungs of the mice after each immunization period; one injection (■), two injections (◆), and three injections (●). Three months after the initial immunization the vaccinated and control mice were challenged with virulent *M. tuberculosis* Erdman by the aerosol route. Five challenge mice were sacrificed following the challenge period and the lung homogenates plated to check the viable counts of the challenge inoculum. Groups of vaccinated and control mice were sacrificed at 14, 28, and 42 days later and the lung and spleen homogenates plated to determine viable colony forming units. Shown in Panel B are the viable challenge bacteria per lung of mice given one dose of the M. tuberculosis lysine auxotroph, and in panel C, the viable challenge bacteria per lung of mice given two doses of the auxotroph. Key: Viable challenge bacteria per lung of mice given the *M. tuberculosis* lysine auxotroph $mc^2$3026 (⊞), BCG-Pasteur (◇), or PBST (○). P values are indicated in the figure. Note that the results shown here are for the lungs. Similar results (not shown) were obtained from the spleens in all the experiments.

FIG. 16 shows a graph summarizing experiments establishing the survival curves of mice immunized three times with the *M. tuberculosis* lysine auxotroph $mc^2$3026. C57Bl/6 mice were injected intravenously with $1x10^6$ CFU of the *M. tuberculosis* lysine auxotroph $mc^2$3026, followed by two more injections at 4 week intervals, and challenged as described in Example 5. The percent survival is shown above for mice immunized thrice with the *M. tuberculosis* lysine auxotroph $mc^2$3026 (■, 5 mice total), once with BCG-Pasteur (◆, 5 mice), and for the PBST controls (●, 10 mice).

FIG. 17 shows graphs summarizing experimental results establishing that the virulence of strain $mc^2$6030 is highly attenuated in SCID mice and BALB/c mice.

FIG. 18 shows graphs summarizing experimental results measuring growth of various strains of *M. tuberculosis* in spleen (Panel A) and lungs (Panel B) of C57BL/6 mice.

FIG. 19 is a graph summarizing experimental results establishing that immunization with $mc^2$6020 and $mc^2$6030 protects mice against TB as effectively as BCG. This graph shows the survival of C57Bl/6 mice challenged with virulent *M. tuberculosis* Erdman through the aerosol route three months after a single dose subcutaneous immunization with either BCG, $mc^2$6020 (Δ*lysA*Δ*panCD*) or $mc^2$6030 (Δ*RD1*Δ*panCD*) and compared to non-immunized naive mice. There were 12 to 15 mice in each survival group.

FIG. 20 is a graph summarizing experimental results establishing that immunization with $mc^2$6030 protects CD4 deficient mice from TB. $CD4^{-/-}$ mice were immunized by a single

subcutaneous injection of 10^6 CFU of either *ΔRD1ΔpanCD* (mc^26030) or BCG-P and three months later challenged with 100-200 CFU of virulent *M. tuberculosis* through the aerosol route and compared to non-immunized (naive) $CD4^{-/-}$ controls. Each group consists of 10 mice.

FIG. 21 shows graphs summarizing experimental results establishing that *M. tuberculosis* double deletion mutants are highly attenuated in SCID mice. A dose of 10^5 mc^26020 or mc^26030 were intravenously inoculated into SCID mice (10 per group) and time to death assessments were performed. While the same dose of *M. tuberculosis* and BCG killed mice in 40 or 90 days, respectively, the mice infected with mc^26020 or mc^26030 survived over 400 or 250 days, respectively.

FIG. 22 is graphs and micrographs of experimental results showing that mc^26030 is severely attenuated in immunocompromised mice. a, Survival of SCID mice infected intravenously with 10^2 CFUs of H37Rv (○) or 10^5 CFUs of mc^26030 (◉). b-c, Bacterial numbers in the spleen (b), lungs (c), and of H37Rv (○) or mc^26030 (◉) infected SCID mice. The results represent means ±standard errors of four to five mice per group. d, Survival of gamma interferon gene-disrupted (GKO) C57BL/6 mice (n = 10 mice) infected intravenously with 10^5 CFUs of H37Rv (○) or mc26030 mutant (●) or M. bovis BCG-P (▲). e, Survival of immunocompetent C57BL/6 mice (n = 10 mice) infected intravenously with 106 CFUs of H37Rv (○) or mc^26030 (●). f-g, Bacterial numbers in spleen (g) and lungs (h) of C57BL/6 mice infected intravenously with H37Rv (○) or mc^26030 (●). The results represent means ±standard errors of four to five mice per group. h, Mild perivascular, lymphocytic infiltrates caused by strain mc26030 in C57BL/6 mice at 3 weeks post-infection. i, Severe granulomatous pneumonia in the lungs of C57BL/6 mice infected with H37Rv at 3 weeks post-infection.

FIG. 23 is graphs of experimental results showing that vaccination with mc26030 induces both short-term and long-term protection in C57BL/6 mice. a-b, Immunocompetent C57BL/6 mice were immunized subcutaneously (s.c) with mc^26030 or BCG-P and challenged with virulent *M. tuberculosis* Erdman through the aerosol route at 3 months after the initial immunization. The CFU numbers reflect the bacterial burden at 28 days post-aerosol challenge in the lungs and spleen of infected mice. c-d, Immunocompetent C57BL/6 mice were immunized subcutaneously (s.c) with mc^26030 or BCG-P and challenged with virulent *M. tuberculosis* Erdman through the aerosol route at 8 months after the initial immunization. The CFU numbers reflect the bacterial burden at 28 days post-aerosol challenge in the lungs and

spleen of infected mice. The results represent means ±standard errors of five mice per group. $**P < 0.01$, $***P < 0.001$ indicate statistical differences between the experimental and unvaccinated control groups. e, Survival of immunocompetent C57BL/6 mice (n = 10 mice) immunized subcutaneously with a single dose of mc²6030 (●) or BCG-P (▲) and challenged 3 months later with virulent M. tuberculosis Erdman through the aerosol route. Unvaccinated mice served as naive controls (○).

FIG. 24 is graphs of experimental results showing that vaccination with mc²6030 protects and confers greater survival advantage to $CD4^{-/-}$ mice from tuberculous challenge. a-b, Protection induced by a single dose of mc²6030 in CD4-deficient mice following aerosol challenge with virulent *M. tuberculosis* Erdman. The CFU numbers reflect the bacterial burden at 28 days post-aerosol challenge in the lungs (a) and spleen (b) from 5 mice per group. $**P < 0.001$ indicate statistical differences between the experimental and unvaccinated control groups. c, Survival of $CD4^{-/-}$ mice (n = 5 or 6 mice) immunized subcutaneously with a single dose of mc²6030 (●) or BCG-P (▲) and challenged 3 months later with virulent M. tuberculosis Erdman through the aerosol route. Unvaccinated mice served as naive controls (○). d-e, Treatment of mc²6030-vaccinated $CD4^{-/-}$ mice with anti-CD8 antibody does not abolish the protection seen in mc²6030-vaccinated control antibody treated $CD4^{-/-}$ mice. The CFU numbers reflect the bacterial burden at 28 days post-aerosol challenge in the lungs (d) and spleen (e) from 5 mice per group. $**P < 0.001$ indicate statistical differences between the experimental and unvaccinated control groups. f, Survival of vaccinated GKO mice following an aerosol challenge with virulent *M. tuberculosis*.

Fig. 25 is micrographs of experimental results showing that. mc²6030 vaccinated $CD4^{-/-}$ mice display improved lung pathology following challenge with virulent M. tuberculosis. a, Severe pneumonia in lung of unvaccinated mice at 28 days post-aerosol challenge, with d, large numbers of *M. tuberculosis* Erdman organisms demonstrated by acid-fast stain. b, Lung from mouse vaccinate with mc²6030 showing milder multifocal areas of pneumonia composed of macrophages and numerous lymphocytes, with e, lower number of *M. tuberculosis* Erdman organisms indicating protection following immunization. c, BCG vaccinated mouse. Similar localized areas of pneumonia adjacent to the airways post-aerosol challenge and f, reduced numbers of acid-fast organisms similar to mc²6030 vaccinated mice.

Detailed Description of the Invention

The present invention is based on the discovery that mycobacteria having two attenuating mutations are safe and protect mammals that are deficient in $CD4^+$ lymphocytes from challenge by virulent mycobacteria. See Examples 6 and 7. This anti-mycobacterial immunity also does not depend on $CD8^+$ cells (Example 7).

Thus, the present invention is directed to methods of treating a mammal that does not have severe combined immune deficiency but is deficient in $CD4^+$ lymphocytes. The methods comprise inoculating the mammal with an attenuated mycobacterium in the *Mycobacterium tuberculosis* (*M. tuberculosis*) complex. In these embodiments, the mycobacterium comprises two deletions, where a virulent mycobacterium in the *M. tuberculosis* complex having either deletion exhibits attenuated virulence. The mammal in these embodiments can have $CD8^+$ lymphocytes that are elevated (as can occur in AIDS patients), normal or deficient (see Example 7).

The protection afforded by those double mutants in mammals deficient in $CD4^+$ lymphocytes is surprising because it was previously believed that $CD4^+$ lymphocytes were crucial in establishing immunity against tuberculosis (Jones et al., 1993; Scanga et al., 2000). The instant discovery establishes that such mutants would be expected to be safe and effective in providing protection against tuberculosis in individuals with highly compromised immunity, such as individuals with HIV infection or those taking immunosuppressant drugs (e.g., transplant patients).

As used herein, a mammal that is deficient in $CD4^+$ lymphocytes has less than about 500 $CD4^+$ cells per mm^3 of blood. The $CD4^+$ deficient mammal can also have less than about 350 or 200 or 100 or 50 cells per mm^3 of blood. The $CD4^+$ deficient mammal can even be devoid of $CD4^+$ lymphocytes. A mammal that is deficient in $CD8^+$ lymphocytes has less than about 200 $CD8^+$ cells per mm^3 of blood. The $CD8^+$ deficient mammal can also have less than about 100 or 50 or 25 $CD8^+$ cells per mm^3 of blood. The $CD4^+$ deficient mammal can also be devoid of $CD4^+$ lymphocytes.

The mammal can be inoculated with the mycobacteria in the methods of the present invention by any of a number of ways known in the art. Non-limiting examples include oral ingestion, gastric intubation, or broncho-nasal-ocular spraying. Other methods of administration include intravenous, intramuscular, intramammary, or, preferably, subcutaneous or intradermal injection. The immunization dosages required can be determined without undue experimentation. One or two dosages of avirulent mycobacteria at 1-2 x 10^6 colony forming

units (CFU) have previously been used, but other dosages are contemplated within the scope of the invention. Multiple dosages can be used as needed to provide the desired level of protection from challenge.

The above-described mycobacterial compositions can be formulated without undue experimentation for administration to a mammal, including humans, as appropriate for the particular application.

Accordingly, the mycobacterial compositions designed for oral, lingual, sublingual, buccal and intrabuccal administration can be made without undue experimentation by means well known in the art, for example with an inert diluent or with an edible carrier. The compositions may be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral administration, the mycobacterial compositions of the present invention may be incorporated with excipients and used in the form of tablets, troches, capsules, elixirs, suspensions, syrups, wafers, chewing gums and the like.

Tablets, pills, capsules, troches and the like may also contain binders, recipients, disintegrating agent, lubricants, sweetening agents, and flavoring agents. Some examples of binders include microcrystalline cellulose, gum tragacanth or gelatin. Examples of excipients include starch or lactose. Some examples of disintegrating agents include alginic acid, corn starch and the like. Examples of lubricants include magnesium stearate or potassium stearate. An example of a glidant is colloidal silicon dioxide. Some examples of sweetening agents include sucrose, saccharin and the like. Examples of flavoring agents include peppermint, methyl salicylate, orange flavoring and the like. Materials used in preparing these various compositions should be pharmaceutically pure and nontoxic in the amounts used.

The mycobacterial compositions of the present invention can easily be administered parenterally such as for example, by intravenous, intramuscular, intrathecal or subcutaneous injection. Parenteral administration can be accomplished by incorporating the mycobacterial compositions of the present invention into a suspension. Such suspensions may also include sterile diluents such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents. Parenteral formulations may also include antibacterial agents such as for example, benzyl alcohol or methyl parabens, antioxidants such as for example, ascorbic acid or sodium bisulfite and chelating agents such as EDTA. Buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose may also be added. The parenteral preparation can be enclosed in ampules, disposable syringes or multiple dose vials made of glass or plastic.

Rectal administration includes administering the mycobacterial compositions into the rectum or large intestine. This can be accomplished using suppositories or enemas. Suppository formulations can easily be made by methods known in the art. For example, suppository formulations can be prepared by heating glycerin to about 120° C., dissolving the composition in the glycerin, mixing the heated glycerin after which purified water may be added, and pouring the hot mixture into a suppository mold.

The present invention also includes nasally administering to the mammal a therapeutically effective amount of the mycobacterial composition. As used herein, nasally administering or nasal administration includes administering the composition to the mucous membranes of the nasal passage or nasal cavity of the patient. As used herein, mycobacterial compositions for nasal administration of a composition include therapeutically effective amounts of the composition prepared by well-known methods to be administered, for example, as a nasal spray, nasal drop, suspension, gel, ointment, cream or powder. Administration of the composition may also take place using a nasal tampon or nasal sponge.

It is well known in the art that in order to elicit an immune response with a live vaccine such as an avirulent mycobacteria, it is preferred that the vaccine organism can sustain an infection in the immunized host, to provide a sustained exposure of the host's immune system to the organism. Therefore, in various preferred embodiments, the mycobacteria used in the methods of the invention are capable of sustaining an infection in the host. The ability to sustain infection can be measured without undue experimentation by any of a number of ways described in the art. With the mycobacteria used in the methods of the present invention, a preferred way of measuring sustained infection is by determining whether viable mycobacteria of the inoculated strain will remain resident in an immunocompetent mouse (e.g., BALB/c or C57BL/6 strain) for more than four weeks. More preferably, the inoculated mycobacteria will remain resident in the mouse for at least ten weeks. In the most preferred embodiments, viable mycobacteria of the inoculated strain will remain resident in the mouse for at least 20 weeks.

Preferably, the attenuated mycobacteria used in the methods of the invention are capable of protecting a mammal from challenge by a virulent *M. tuberculosis* complex mycobacteria. This ability can be determined by any of a number of ways provided in the literature. A preferred method is aerogenically treating an immunocompetent mouse with the virulent mycobacteria, as described in Examples 1 and 2. Aerogenic challenge is preferred because that most closely mimics natural infection. The skilled artisan would understand that the ability of an avirulent mycobacterium to protect a mouse from challenge from a virulent

mycobacterium is indicative of the ability of the avirulent mycobacterium to protect a human, including a human child, from tuberculosis infection. A more stringent test of an avirulent mycobacterium to prevent infection by a virulent challenge is to use an immunocompromised mammal, e.g. a SCID mouse or a mouse deficient in CD4 or interferon γ production.

The scope of the present invention includes the use of mycobacteria in the *M. tuberculosis* complex that comprise two attenuating deletions, where at least one of the attenuating deletion was made using genetic engineering. As discussed above, examples of such deletions include deletions of an RD1 region, deletions of a region controlling production of a vitamin, and deletions of a region controlling production of an amino acid. These mycobacteria include any in the *M. tuberculosis* complex, including *M. africanum, M. bovis* including the BCG strain and the subspecies *caprae, M. canettii, M. microti, M. tuberculosis* and any other mycobacteria within the *M. tuberculosis* complex, now known or later discovered. Preferred species are *M. bovis*, including the BCG strain, and *M. tuberculosis*, since those species are the most important as causes of mammalian diseases, such as tuberculosis in humans and *M. bovis* infection in cows.

In some aspects of these embodiments, at least one of the two deletions is of the *RD1* region (see Example 1). Strains with these deletions can be determined by any means in the art, preferably by molecular genetic means, for example by hybridization methods (e.g., Southern blot using a probe from the *RD1* region) or by amplification methods (e.g., PCR using primers to amplify a portion of the *RD1* region). An example of an *M. tuberculosis RD1* region (from H37Rv) is provided herein as SEQ ID NO:1. The skilled artisan could identify analogous *RD1* regions from other mycobacteria in the *M. tuberculosis* complex without undue experimentation. Those *RD1* regions would be expected to have strong homology to SEQ ID NO:1, at least 80% homologous to SEQ ID NO:1. However, it is to be understood that virulent mycobacteria in the *M. tuberculosis* complex can be rendered avirulent by deletions in a portion of the *RD1* region. Therefore, use of non-naturally occurring mycobacteria in the *M. tuberculosis* complex that have a partial deletion in the *RD1* region are envisioned as within the scope of the invention, provided the deletion can cause a virulent *M. tuberculosis* to become avirulent. It is expected that such *M. tuberculosis* with partial *RD1* deletions can still sustain an infection in a mammal and protect against challenge by a virulent *M. tuberculosis*.

In embodiments where at least one of the deletions is in a region controlling production of a vitamin, the deletion can be in any genetic element leading to loss of

production of the vitamin, including structural genes for enzymes involved in the biosynthesis of the vitamin, and genetic control elements such as promoters, enhancers, etc.

Deletion of a region controlling production of any essential vitamin or their precursors is contemplated as within the scope of the invention. As used herein, an essential vitamin is defined by its normal usage, that is, a small molecular weight compound that is required as a cofactor for the efficient function of an essential enzyme or enzymes. Nonlimiting examples include vitamin A, thiamin (B1), riboflavin (B2), nicotinic acid (niacin)/ nicotinamide/ nicotinamide adenine dinucleotide (NAD)/ nicotinamide adenine dinucleotide phosphate (NADP/coenzyme II), pantothenate (pantothenic acid/B5), pyridoxine (B6), folic acid, B12, biotin, C, D, E and K. Preferred vitamin targets for deletion include nicotinamide and pantothenate (see Example 2). Methods for determining whether a mycobacterium has deletions leading to the loss of production of any of these vitamins are within the scope of the art.

Deletions leading to the loss of any of these vitamins would be expected to lead to attenuated virulence of an otherwise virulent mycobacterium in the *M. tuberculosis* complex. Any of those strains would also be expected to sustain an infection in a mammal.

Preferred vitamin targets are pantothenate and nicotinamide adenine dinucleotide (NAD)(see Example 2). A preferred pantothenate deletion is of structural genes in the pantothenate biosynthetic operon, most preferably the pan C and D genes, the combined mutation being Δ*panCD*. An example of a deletion of those genes is the deletion of the sequence from *M. tuberculosis* H37Rv provided herein as SEQ ID NO:2. Similarly, a preferred NAD deletion is in the structural genes of the NAD biosynthetic operon, most preferably the *nadB* and *nadC* genes, the combined mutation being Δ*nadBC*. An example of a deletion in those genes is the deletion of the sequence from *M. tuberculosis* H37Rv provided herein as SEQ ID NO:3.

In other embodiments, at least one of the attenuating deletions is of a region controlling production of an amino acid. Preferred examples include deletions of a region controlling production of proline, tryptophan, leucine or lysine. When the amino acid is lysine, a preferred deletion is a Δ*lysA* deletion, e.g., SEQ ID NO:4.

The mycobacterium deletions can be made by serial in vitro passage of a virulent *M. tuberculosis* (as the well-known *M. bovis* BCG was made) and selection for the desired deletion. More preferably, however, the deletion is made by genetic engineering, since such genetic methods allow precise control of the deletion being made.

Various methods of making deletions in mycobacteria are known in the art. Nonlimiting examples include specialized transduction (see, e.g., U.S. Patent No. 6,271,034, Example 1 and Example 2), and sequential two-step recombination (see Example 1). The latter method can usefully employ a a *sacB* selective marker (Example 1).

Since, in preferred embodiments of the invention, the mycobacteria exhibit attenuated virulence and can sustain an infection in a mammal, these mycobacteria can usefully further employ a foreign DNA stably integrated into the genome of the mycobacteria, such that the mycobacteria can express a gene product coded by the foreign DNA. See, e.g., U.S. Patent 5,504,005.

Thus, the present invention has wide applicability to the development of effective recombinant vaccines in mammals deficient in $CD4^+$ lymphocytes against bacterial, fungal, parasite or viral disease agents in which local immunity is important and might be a first line of defense. Non-limiting examples are recombinant vaccines for the control of bubonic plague caused by *Yersinia pestis*, of gonorrhea caused by *Neisseria gonorrhoea*, of syphilis caused by *Treponema pallidum*, and of venereal diseases or eye infections caused by *Chlamydia trachomatis*. Species of *Streptococcus* from both group A and group B, such as those species that cause sore throat or heart disease, *Neisseria meningitidis*, *Mycoplasma pneumoniae*, *Haemophilus influenzae*, *Bordetella pertussis*, *Mycobacterium leprae*, *Streptococcus pneumoniae*, *Brucella abortus*, *Vibrio cholerae*, *Shigella* spp., *Legionella pneumophila*, *Borrelia burgdorferi*, *Rickettsia* spp., *Pseudomonas aeruginosa*, and pathogenic *E. coli* such as ETEC, EPEC, UTEC, EHEC, and EIEC strains are additional examples of microbes within the scope of this invention from which foreign genes could be obtained for insertion into mycobacteria of the invention. Recombinant anti-viral vaccines, such as those produced against influenza viruses, are also encompassed by this invention. Recombinant anti-viral vaccines can also be produced against viruses, including RNA viruses such as Picornaviridae, Caliciviridae, Togaviridae, Flaviviridae, Coronaviridae, Rhabdoviridae, Filoviridae, Paramyxoviridae, Orthomyxoviridae, Bunyaviridae, Arenaviridae, Reoviridae or Retroviridae; or DNA viruses such as Hepadnaviridae, Paroviridae, Papovaviridae, Adenoviridae, Herpesviridae or Poxviridae.

The use of these methods for administering recombinant vaccines to protect against infection by pathogenic fungi, protozoa or parasites are also contemplated by this invention.

The avirulent microbes used in the methods of the present invention are also contemplated for use to deliver and produce foreign genes that encode pharmacologically

active products that might stimulate or suppress various physiological functions (i.e., growth rate, immune-stimulating cytokines, blood pressure, etc.) in CD4$^+$-deficient mammals. In such microbes, the recombinant gene encodes said pharmacologically active products.

By immunogenic agent is meant an agent used to stimulate the immune system of an individual, so that one or more functions of the immune system are increased and directed towards the immunogenic agent. Immunogenic agents include vaccines.

An antigen or immunogen is intended to mean a molecule containing one or more epitopes that can stimulate a host immune system to make a secretory, humoral and/or cellular immune response specific to that antigen.

In preferred embodiments, the foreign DNA encodes an antigen, an enzyme, a lymphokine, an immunopotentiator, or a reporter molecule. Preferred examples include antigens from *Mycobacterium leprae*, *Mycobacterium tuberculosis*, malaria sporozoites, malaria merozoites, diphtheria toxoid, tetanus toxoids, *Leishmania spp.*, *Salmonella spp.*, *Mycobacterium africanum*, *Mycobacterium intracellulare*, *Mycobacterium avium*, *Treponema spp.*, Pertussis, Herpes virus, Measles virus, Mumps virus, *Shigella spp.*, *Neisseria spp.*, *Borrelia spp.*, rabies, polio virus, human immunodeficiency virus, snake venom, insect venom, and *Vibrio cholera*; steroid enzymes; interleukins 1 through 7; tumor necrosis factor α and β; interferon α, β, and γ; and reporter molecules luciferase, β-galactosidase, β-glucuronidase and catechol dehydrogenase.

It has also been discovered that the attenuated mycobacteria discussed above can safely be used to inoculate mammals deficient in CD8$^+$ lymphocytes, for example mammals that are immunosuppressed, or mammals that have a mutation in a CD8 gene (see, e.g., de la Calle-Marin et al., 2001). See Example 7.

Thus, the invention is also directed to methods of treating a mammal that does not have severe combined immune deficiency but is deficient in CD8$^+$ lymphocytes. The methods comprise inoculating the mammal with an attenuated mycobacterium in the *Mycobacterium tuberculosis* (*M. tuberculosis*) complex. In these embodiments, the mycobacterium comprises two deletions, where a virulent mycobacterium in the *M. tuberculosis* complex having either deletion exhibits attenuated virulence. The mammals in these methods can have normal levels of CD4$^+$ lymphocytes, or have deficient or elevated levels of CD4$^+$ lymphocytes.

These embodiments are entirely analogous to the embodiments discussed above relating to the treatment of mammals deficient in CD4$^+$ lymphocytes. This includes the use of any of the above-described attenuated *M. tuberculosis* strains and any of the inoculation

methods described above. The methods can also be used in any mammal, including humans, such as adults or children.

Additionally, the invention is directed to the use of an attenuated mycobacterium in the *Mycobacterium tuberculosis* (*M. tuberculosis*) complex for the manufacture of a medicament for treatment of a mammal that does not have severe combined immune deficiency but is deficient in $CD4^+$ lymphocytes, or $CD8^+$ lymphocytes. In these embodiments, the mycobacterium is as described for the embodiments discussed above, i.e., it comprises two deletions, where a virulent mycobacterium in the *M. tuberculosis* complex having either deletion exhibits attenuated virulence. Methods of manufacture of such medicaments, including formulations as vaccines, are well known in the art and would not require undue experimentation. These uses are suitable for any mammal, including humans, such as adults or children.

Preferred embodiments of the invention are described in the following examples. Other embodiments within the scope of the claims herein will be apparent to one skilled in the art from consideration of the specification or practice of the invention as disclosed herein. It is intended that the specification, together with the examples, be considered exemplary only, with the scope and spirit of the invention being indicated by the claims which follow the examples.

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual" (1989); "Current Protocols in Molecular Biology" Volumes I-IV Ausubel, R. M., ed. (1997); and "Cell Biology: A Laboratory Handbook" Volumes I-III J. E. Celis, ed. (1994).

Example 1. *Mycobacterium tuberculosis* having an *RD1* deletion has attenuated virulence and protects against tuberculosis

This example describes experimental methods and results that establish that deleting the *RD1* region from a virulent *M. tuberculosis* attenuates the virulence of the *M. tuberculosis* in both immunocompetent and immunocompromised mice, and protects against subsequent challenge by a virulent *M. tuberculosis*.

Materials and Methods

Media and Cultures. The mycobacterial strains *M. tuberculosis* H37Rv, *M. tuberculosis* Erdman and *M. bovis* BCG Pasteur were obtained from the Trudeau Culture Collection (Saranac Lake, NY). They were cultured in Middlebrook 7H9 broth and 7H10 agar supplemented with 10% OADC, 0.5% glycerol, and 0.05% Tween 80. Cyclohexamide, which does not affect mycobacterial growth, was added to the 7H10 agar medium at 0.1% to avoid fungal contamination. To examine the colony morphology of mycobacteria, Tween 80 was not added to 7H10 agar medium. The acriflavin resistant strain (Hepper and Collins, 1984) of *M. tuberculosis* Erdman grew in the presence of 20 μg of acriflavin per ml of medium.

DNA manipulation and construction of *M tuberculosis ΔRD1*. The following four primers were used to amplify upstream and downstream flanking sequences (UFS and DFS, respectively) for the construction of the *RD1* deletion mutants. UFS was amplified using TH201: GGGGCGCACCTCAAACC (SEQ ID NO:5) and TH202: ATGTGCCAATCGTCGACCAGAA (SEQ ID NO:6). DFS was amplified using TH203: CACCCAGCCGCCCGGAT (SEQ ID NO:7), and TH204: TTCCTGATGCCGCCGTCTGA (SEQ ID NO:8). Recognition sequences for different restriction enzymes were included at the ends of each primer to enable easier manipulation.

The unmarked deletion mutant of *M. tuberculosis* H37Rv, mc^{2}4004, was generated by transformation (Snapper et al., 1988) using a *sacB* counterselection (Pelocic et al., 1996; Pavelka and Jacobs, 1999). Specifically, the plasmid pJH508 was created by first cloning UFS into *Kpn*I and *Xba*I sites, then cloning DFS into *EcoR*I and *Hind*III sites of pJH12, a pMV261-derived *E coli*-Mycobacteria shuttle plasmid, to create pJH506 in which UFS and DFS flanked a green fluorescent protein gene (GFPuv, Clonetech) whose expression was driven by the *M. leprae* promoter 18Kd. The UFS-gfp-DFS cassette was sub-cloned into the *EcoR*V site of plasmid pYUB657 to create pJH508. The first homologous recombination involved the identification of hygromycin resistant colonies, resulting from the transformation of *M. tuberculosis* with pJH508. Southern analysis of the *Nco*I digested DNA isolated from hygromycin resistant colonies probed with UFS or DFS, confirmed the presence of a single copy of pJH508 inserted into the *M. tuberculosis* genome. The transformant identified was then grown in 7H9 broth to saturation, to allow the second homologous recombination to occur, resulting in recombinants that could be selected by plating the culture on 7H10 plates, supplemented with 3% sucrose. Both Southern analysis and PCR of the DNA isolated from sucrose resistant colonies confirmed the RD1 deletion.

Specialized transduction (Bardarov and Jacobs, 1999), a mycobacteriophage-based method for the delivery of homologous DNA constructs using conditionally replicating shuttle phasmids (Jacobs et al, 1987; Bardarov and Jacobs, 1999; Carriere et al., 1997), has been used successfully for *M. tuberculosis* (Glickman et al., 2000; Glickman et al., 2001; Raman et al., 2001). Specifically, a transducing phage phAEKO1 was constructed by inserting UFS and DFS into pJSC347, flanking a hygromycin cassette, to create pJH313. pJH313 was digested with *Pac*I and ligated to phAE159, a temperature sensitive mycobacteriophage derived from TM4. The transduction was performed by growing *M. tuberculosis* to an $O.D._{600}$ of 0.8, washing twice with MP buffer, re-suspending into an equal volume of MP buffer and mixing with the transducing phage phAEKO1 at an MOI of 10. The mixtures were incubated at 37°C overnight, then plated on 7H10 plates supplemented with hygromycin at 50 μg/ml. Hygromycin resistant colonies were analyzed by PCR and Southern hybridization, as described above, to confirm the deletion of RD1.

Complemetation analyses was performed using the integration proficient cosmids (Pascopella et al., 1994; Lee et al., 1991) pYUB412 made by S. Bardarov, a library made by F. Bange, and cosmid identified and generously provided by S.T. Cole.

Results

Genetic engineering of *M. tuberculosis* mutants with *RD1* deletions. The *RD1* (region of difference) region has been defined as the specific 9454 bp of DNA that is present in virulent *M. tuberculosis* and *M. bovis*, but absent in BCG (Mahairas et al., 1996). The annotation of *RD1* predicts that the deletion would disrupt 9 genes encoding ORF's (Id.; Cole et al., 1998). Five of the 9 ORF's have no known functions (Rv3871, Rv3876, Rv3877, Rv3878 and Rv3879c), two genes encode members of the PE/PPE family (Rv3872/Rv3873), and two genes encode the secreted proteins Cfp10 (Berhet et al., 1998) and Esat6 (Andersen et al., 1991)(Rv3875) (Fig. 1). To test if the *RD1* region is essential for virulence in *M. tuberculosis*, it was necessary to 1) delete the *RD1* region from virulent *M. tuberculosis* strains, 2) demonstrate loss of virulence and 3) restore virulence by complementation with the *RD1* DNA. The *RD1* deletion (Δ*RD1*) was successfully introduced into *M. tuberculosis* by two different techniques, utilizing both a plasmid that allows two-step sequential recombination to make an unmarked deletion, and specialized transduction (Fig. 1a-c). For both methods, the same 1200 bp on each side of the *RD1* deletion were cloned into the appropriate plasmid or phage vector and then introduced into *M. tuberculosis* H37Rv by transformation or phage infection. An unmarked *RD1* deletion mutant of *M. tuberculosis* H37Rv, mc^24004, was

constructed, purified, and has the advantage that additional mutations can be readily added to it. In addition, the *RD1* deletion was successfully engineered in the H37Rv and Erdman strains of *M. tuberculosis* using a specialized transducing phage. Since TM4 phages have been shown to infect over 500 clinical *M. tuberculosis* isolates (Jacobs et al., 1987), it should be possible to introduce the *RD1* deletion into any *M. tuberculosis* isolate of interest.

M. tuberculosis H37Rv Δ*RD1* is attenuated for virulence. To test if the *RD1* deletion causes an attenuating phenotype in *M. tuberculosis*, the *M. tuberculosis* H37Rv Δ*RD1* ($mc^2$4004) was introduced into immunocompromised mice possessing the SCID (severe combined immunodeficiency) mutation. Groups of ten mice were injected intravenously with either 2×10^6 *M. tuberculosis* H37Rv or *M. tuberculosis* H37Rv Δ*RD1* and three mice per group were sacrificed 24 hours later to verify the inoculation doses. All of the SCID mice infected with the parental *M. tuberculosis* H37Rv strain died within 14 to 17 days post infection (Fig. 2a). In contrast, the SCID mice infected with the same dose of *M. tuberculosis* H37Rv Δ*RD1* were all alive at 35 days post-infection demonstrating a marked attenuation of the strain. To prove that the attenuation was due to the *RD1* deletion, $mc^2$4004 was transformed with an integrating plasmid containing the *RD1* region from *M. tuberculosis* H37Rv. SCID mice injected intravenously with 2×10^6 of the transformed strain died 13 to 16 days post-infection (Fig. 2a), thereby, establishing that the genes in the RD1 deletion complemented the attenuating phenotype.

To further characterize the attenuating phenotype of the *RD1* deletion in $mc^2$4004, we compared the virulence of *M. tuberculosis* H37Rv and BCG-Pasteur to *M. tuberculosis* H37Rv Δ*RD1* with time-to-death experiments in SCID mice following injections with 10-fold varying inocula. Groups of 10 mice were injected intravenously, each mouse receiving from 2×10^3 to 2×10^6 CFU. Fig. 2b shows that the SCID mice succumbed to the infection with all three mycobacterial strains. However, the SCID mice succumbed to an *M. tuberculosis* H37Rv intravenous infection within 2 to 5 weeks, in a dose dependent manner. In the same time frame, the SCID mice did not succumb to infection with *M. tuberculosis* H37Rv Δ*RD1* until week 7, and only then, with the high dose of 2×10^6 CFU. Mice receiving 2×10^3 CFU *M. tuberculosis* H37Rv Δ*RD1* survived longer than 14 weeks post infection, the survival rate of which coincided with the mice receiving 2×10^6 CFU of *M. bovis* BCG. Thus, these experiments established that *M. tuberculosis* H37Rv Δ*RD1* was significantly more attenuated than its parent, but not as attenuated as BCG-Pasteur in the immunocompromised mice.

Colonial morphotypes of *M. tuberculosis* H37Rv Δ*RD1*. The *M. tuberculosis* H37Rv Δ*RD1* mutant was generated independently three times from the single crossover construct (mc^24000) and upon subculturing, consistently yielded a 20 to 50% mixture of two colonial morphotypes on Middlebrook medium without Tween 80 (Fig. 3a). One morphotype was a smooth (S) phenotype that was flat and corded (like the parental *M. tuberculosis* H37Rv strain) and the second was a rough and raised (R) phenotype. Repeated subculturing of either the R or S colonies continued to yield both colonial morphotypes, but with a distribution of approximately 80% smooth and 20% rough colonies. The distinction of these two types of morphology could be noted even when the colonies were less than two weeks old as the rough colonies were constricted and elevated with only a small portion of the base of the colony attached to the agar, while the smooth colonies tends to be flattened and spread out. When colonies grew older, e.g. 6 weeks old, the rough colonies remained more constricted compared to those of smooth colonies. The rough colonies exhibited large folds on the surface (Fig. 3f, g), as compared to those of the smooth colonies that exhibited small wrinkles (Fig. 3e).

Interestingly, in 1929, Petroff *et al.* reported a similar property for an early-derived BCG strain (Petroff et al., 1929) and proposed that the attenuation phenotype of BCG was not stable. Calmette disputed that the avirulent phenotype reverted and postulated that Petroff et al. had acquired a contaminating virulent strain. Southern analysis of R and S colonies revealed each morphotype has the same *RD1*-deleted genotype (Fig. 3d). Furthermore, complementation of *M. tuberculosis* H37Rv Δ*RD1* with the RD1 region restored the mutant phenotype back to the homogenous parental S phenotype (Fig. 3a-c). These results suggest that the variable morphotypes resulted directly from the RD1 deletion thus dissociating a direct correlation of virulence with morphotype.

The *M. tuberculosis* H37Rv Δ*RD1* is highly attenuated in immunocompetent BALB/c mice. To further assess the pathogenicity, survival, growth kinetics, and the histopathological analysis of the *M. tuberculosis* H37Rv Δ*RD1* mutant, we compared the parental *M. tuberculosis* H37Rv to BCG-Pasteur strains in BALB/c mice. In survival studies, greater than 50% BALB/c mice had died at 14 weeks post i.v. infection with $2x10^6$ CFUs of *M. tuberculoisis* H37Rv strain (Fig. 4a). In contrast, all mice infected with a similar dose of either BCG or *M. tuberculosis* H37Rv Δ*RD1* survived for longer than 22 weeks. These results were substantiated in a separate experiment in which a group of 11 BALB/c mice were infected with $1X10^5$ CFU of *M. tuberculosis* H37Rv Δ*RD1* and 9 of 11 mice (81%) survived greater than 9 months post-infection (data not shown). While BCG and *M. tuberculosis* H37Rv Δ*RD1*

showed similar survival results, the growth relative kinetics in mouse organs differed substantially. BCG grew in a limited fashion in lungs, liver and spleen in BALB/c mice and was cleared to undetectable levels by week 12 (Fig. 4b-d). In contrast, the *M. tuberculosis* H37Rv Δ*RD1* strain grew in a fashion indistinguishable from the parental *M. tuberculosis* H37Rv in all mouse organs for the first 8 weeks. Thereafter, mice infected with the parental *M. tuberculosis* failed to contain the infection leading to mortality. Strikingly, mice infected with the *M. tuberculosis* H37Rv Δ*RD1* showed a definite control over infection resulting in significantly prolonged survival of mice (Fig. 4b-d).

The differing survival data of the three strains was clearly substantiated by histopathological analysis. *M. tuberculosis* H37Rv Δ*RD1* caused less severe organ damage in the lung, liver and spleen than the highly virulent parent strain *M. tuberculosis* H37Rv. *M. bovis* BCG was the least virulent of the three strains. Based on histopathological evaluation, the mortality in mice infected with the wild type *M. tuberculosis* H37Rv (documented above and in Fig. 4a) was caused by worsening pneumonia, hepatitis and splenitis (Fig. 5a-c). Mice examined at 14 weeks post-infection had developed severe lobar granulomatous pneumonia. Acid fast staining demonstrated large numbers of *M. tuberculosis* H37Rv, often in clumps, throughout the lung. The livers and spleens showed a severe diffuse granulomatous inflammation.

Histopathological examination further demonstrated that *M. tuberculosis* H37Rv Δ*RD1* was attenuated in virulence compared to the parent strain *M. tuberculosis* H37Rv (Fig. 5d-f). In contrast to the rapidly progressive infection with the parent strain *M. tuberculosis* H37Rv, the lung lesions caused by *M. tuberculosis* H37Rv Δ*RD1* were maximal in mice examined at 8 weeks post-infection. Consolidating granulomatous pneumonia involved an estimated 25-30% of the lung in these mice. Numerous organisms were demonstrated by acid fast staining. The pneumonia subsequently underwent partial resolution. By 14 weeks, and again at 22 weeks post-infection, the lungs showed peribronchial and perivascular inflammatory cell accumulations and focal, generally non-confluent, granulomas now with a prominent lymphocytic infiltration. The numbers of acid fast organisms were reduced. Liver lesions consisted of low numbers of scattered granulomas. Spleens were smaller, with persistent granulomas in the red pulp.

Mice infected with *M. bovis* BCG showed mild lesions in the lung, liver and spleen at all time points (Fig. 5g-i). At longer time intervals post-infection the lesions were fewer in number, smaller with prominent lymphocytic infiltrations. At 14 weeks post-infection, *M.*

bovis BCG was below the level of detection by acid fast staining. In summary, whereas *M. tuberculosis* H37Rv Δ*RD1* initially grew in a manner similar to the parental *M. tuberculosis* H37Rv, this *RD1* mutant was limited in the extent of spread of infection, particularly in the lung. This contrasted to the extensive and severe damage caused by the parent strain. The subsequent resolving granulomas, localization of the lesions and changes in the composition of the inflammatory cell infiltrations suggested that the mice mounted an effective immune response to combat *M. tuberculosis* H37Rv Δ*RD1* infection and thereby reduced the numbers of viable organisms.

<u>*M. tuberculosis* H37Rv Δ*RD1* protects mice against aerosolized *M. tuberculosis* challenge</u>. To test the potential of *M. tuberculosis* H37Rv Δ*RD1* to immunize mice and protect against tuberculous challenge, we used the model of subcutaneous immunization followed by aerosol challenge with virulent *M. tuberculosis*. Our initial studies in C57BL/6 mice monitored the growth the *M. tuberculosis* H37Rv Δ*RD1* strain over an 84-day period. Groups of mice (5 mice per group) were vaccinated subcutaneously (sc) either once or twice, 6 weeks apart, with 10^6 CFU *M. tuberculosis* H37Rv Δ*RD1* organisms. Additional mice were infected intravenously (iv) with the same dose of the RD1-deleted strain in order to examine the pathogenicity in C57BL/6 mice.

As seen in Table 1, *M. tuberculosis* H37Rv Δ*RD1* persisted in the lungs, liver, and spleen for 3 months at moderate levels of infection but the organisms failed to grow substantially in the lungs and spleens of mice that had been inoculated iv. In contrast, reduced persistence and decreased concentrations of *M. tuberculosis* H37Rv Δ*RD1* organisms were detected in organ homogenates prepared from mice that had been vaccinated sc. For the groups of mice that had been immunized with only one dose sc., low levels of *M. tuberculosis* H37Rv Δ*RD1* bacilli were recovered from the spleen after 28 and 56 days post-vaccination; however, no splenic mycobacteria were detected 84 days after the sc. injection. Importantly, the concentration of *M. tuberculosis* H37Rv Δ*RD1* organisms in the lungs after the sc. immunizations was below the threshold of detection (<100 CFUs per organ) for the CFU assay at nearly all time points during the three month study.

Table 1. Growth kinetics in C57BL/6 mice.

	Lung (Log CFU)			Spleen (Log CFU)		
Weeks	i.v.	s.c.	s.c. (2X)	i.v.	s.c.	s.c. (2X)
4	5.86±0.10	<2	not done	5.73±0.05	2.41±0.26	not done
8	5.79±0.07	<2	2.52±0.34	5.37±0.04	3.12±0.40	3.62±0.29
12	5.61±0.09	<2	<2	5.40±0.05	<2	3.52±0.22

Mice were infected with 10^6 *M. tuberculosis* H73Rv Δ*RD1* by different routes. The data are presented as mean ± standard error of the mean.

Three months after the sc. vaccinations with the Δ*RD1* strain, groups of mice were challenged aerogenically with a low dose (50 CFUs) of an acriflavin-resistant strain of *M. tuberculosis* Erdman. The use of a drug-resistant challenge strain permitted the differentiation of the challenge organisms from the sensitive vaccine population. As controls, other groups of mice were immunized sc. with 10^6 CFUs of BCG Pasteur. The protective responses induced by the *M. tuberculosis* H37Rv Δ*RD1* vaccination were evaluated by assessing the relative growth of the acriflavin-resistant challenge organisms in naïve, BCG vaccinated, and *M. tuberculosis* H37Rv Δ*RD1* immunized mice and by comparing the relative post-challenge lung pathology in the experimental groups and the naive controls. As seen in Table 2, the growth of the drug-resistant challenge organisms was substantially lower in the lungs of animals vaccinated with BCG or the *M. tuberculosis* H37Rv Δ*RD1* vaccine. Significant reductions in the lung CFU values in the vaccinated animals (relative to naive controls) could be detected both 28 and 56 days after the challenge. Dissemination to the spleen was also significantly limited in all of the vaccination groups with the most substantial differences (-1.4 $\log_{10}$ CFUs compared to the naives) being detected during the first month post-challenge. While significant differences in the growth of the mycobacterial challenge was identified between unvaccinated and vaccinated mice, the rate of proliferation of the acriflavin-resistant challenge strain in all the experimental groups (BCG sc or *M. tuberculosis* H37Rv Δ*RD1* 1 or 2 doses sc) was nearly identical and not statistically different.

Table 2. *M. tuberculosis* Δ*RD1* and BCG protect C57BL/6 mice from areosol challenge with *M. tuberculosis* Erdman

	Lung (Log CFU)		Spleen (Log CFU)	
	Day 28	Day 56	Day 28	Day 56
Naive	4.77±0.06	4.11±0.05	3.57±0.21	3.20±0.16
BCG (1X)	3.96±0.20	3.80±0.08	2.18±0.18	2.48±0.23
ΔRD1 (1X)	3.97±0.39	3.71±0.06	2.12±0.12	2.60±0.25
ΔRD1 (2X)	3.96±0.15	3.66±0.09	2.21±0.15	2.22±0.16

Immunizations were performed subcutaneously once (1X) or twice (2X) with $2x10^6$ CFUs of the vaccinating strains. Three months later, vaccinated animals were aerogenically challenged with 50 CFUs/mouse of acriflavin resistant *M. tuberculosis* Erdman. The growth of the bacterial challenge was monitored 28 and 56 days post infection by plating on Middlebrook 7H11 plates containing 20μg/ml acriflavin and using procedures previously described (Delogu et al., 2002).

Discussion

The *M. tuberculosis* H37Rv Δ*RD1* mutant strain shares significant properties with BCG including: 1) a significant attenuation of virulence in mice, 2) the ability to generate variable colonial morphotypes, and 3) the ability to protect mice against aerogenic tuberculosis challenge. These properties, and the observation that *RD1* is the only deletion common to all BCG substrains, makes it likely that the *RD1* deletion is the primary attenuating mutation. It remains to be determined if a single gene or a number of genes in this region causes the attenuated phenotype. The variable colonial morphotype switch does suggest that a protein regulating cell wall biogenesis is affected. Notably, defined mutations affecting the cyclopropanation of mycolic acids (Glickman et al., 2000) or the synthesis or export of phthiocerol dimycoseroate (Cox et al., 1999) have been found to correlate with decreased virulence and altered colony morphotypes in *M. tuberculosis* and thus represent attractive candidate genes that might be regulated by an *RD1*-encoded gene. The *M. tuberculosis* Δ*RD1* mutant provides a precisely defined background strain by which to determine virulence and colony morphology related genes.

BCG is currently the only antituberculous vaccine available for use in humans. In many animal models, BCG has been shown to induce protective immunity against *M. tuberculosis* challenge (Opie and Freund, 1937; Hubbard et al., 1992; Baldwin et al., 1998) and in addition, has demonstrated protection against the most severe and fatal form of TB in children (Rodrigues et al., 1991). However, BCG has shown variable efficacy in protecting

adults from pulmonary TB (Tuberculosis Prevention Trial, 1980; Hart and Sutherland, 1977; Bloom and Fine, 1994). Due to the uncertain efficacy of BCG, particularly in TB-endemic countries, the development of improved tuberculosis vaccines has become an international research priority.

Our challenge studies have demonstrated that the protective immune responses elicited by immunization with *M. tuberculosis* H37Rv Δ*RD1* in mice are at least as strong as the protective responses induced by vaccination with BCG. The *M. tuberculosis* H37Rv Δ*RD1* mutant also retains the BCG-associated property of limited spread to the lung following subcutaneous immunization. Restricted dissemination of the Δ*RD1* mutant to the lung suggests it should have a favorable overall safety profile. Also, the unmarked mutant of *M. tuberculosis* H37Rv Δ*RD1* provides a single deletion strain whereby other attenuating mutations can be readily engineered. Since the risk of reversion to wild-type virulence decreases substantially with each additional attenuating mutation, *M. tuberculosis* mutants harboring deletions in two or three separate genetic loci should provide a much safer vaccine for long term use.

M. tuberculosis mutants with *RD1* deletions represent attractive candidates as novel vaccines for TB prevention. These mutants, derived from a single mutagenic event from the parental *M. tuberculosis* strain, replicate more efficiently *in vivo* than BCG, especially early in infection. This enhanced rate of proliferation for the *RD1*-deleted strains, relative to BCG, may lead to the induction of increased protective immunity in humans, after vaccination with *M. tuberculosis* H37Rv Δ*RD1*. Moreover, they could also be more immunogenic as there exist at least 129 ORFs present in *M. tuberculosis* H37Rv that are absent from *M. bovis* (Behr et al., 1999). Since some of these ORFs are likely to encode regulatory proteins affecting the expression of other genes, there could be hundreds of antigens expressed in *M. tuberculosis*-infected cells that are absent from BCG-infected cells. Thus, *RD1* deletion mutants constructed from human tubercle bacilli could protect humans against disease substantially better than BCG.

Example 2. Vitamin auxotrophs of *Mycobacterium tuberculosis* are attenuated and protect against tuberculosis

This example describes experimental methods and results that establish that deleting genes that control vitamin production in a virulent *M. tuberculosis* causes the *M.*

tuberculosis to become avirulent and sustain an infection in mammals, and protect the mammal against challenge with a virulent *M. tuberculosis*.

Given the importance of NAD and nicotinamide (vitamin B3) and pantothenate (vitamin B5) as cofactors involved in carbon utilization, energy transduction (Abiko, 1975; Jackowski, 1996) and the biosynthesis of the complex lipid cell wall of *M. tuberculosis*, we hypothesized that mutations in the biosynthetic pathways for NAD and pantothenate could lead to the generation of mutant strains that retain a limited ability to replicate and subsequently get cleared within the host tissues. In *M. tuberculosis*, the *nadABC* operon controls the *de novo* biosynthesis of NAD. Similarly, the *panC* and *panD* genes that are organized in an operon control the rate-limiting step in the *de novo* biosynthesis of pantothenate. We constructed deletion mutants of *M. tuberculosis* in the *nadBC* and *panCD* genes using specialized transduction, as described in Example 1. The mutant strains $mc^2$3122 (Δ*nadBC*) and $mc^2$6001 (Δ*panCD*) were auxotrophic for nicotinamide and pantothenate respectively. The *in vitro* reversion frequencies of the respective mutations were found to be less than 10^{-10} events per generation.

The safety and attenuation of Δ*nadBC* and Δ*panCD* auxotrophic mutants were assessed by infection of immune-compromised SCID mice. SCID mice infected with wild-type *M. tuberculosis* and the Δ*nadBC* mutant succumbed to infection in about 5 weeks (data not shown). This result clearly indicates that in the absence of T-cell immunity, intermediates of NAD biosynthetic pathway, such as nicotinamide, are readily available in the macrophages to support the growth of the Δ*nadBC* mutant. In contrast all mice infected with the Δ*panCD* mutant survived longer than 30 weeks, demonstrating the severe attenuation of this mutant strain. The full virulence phenotype was restored when the *panCD* wild type alleles were integrated into the chromosome of the Δ*panCD* mutant in single copy, suggesting the observed attenuation in Δ*panCD* to be due to the requirement of pantothenate for growth and not due to polar effects of the mutation on downstream genes. SCID mice infected with the same dose of conventional BCG-Pasteur vaccine strain succumbed to infection within 80 days (Fig. 6A) in accordance with earlier reports (Guleria, 1996). Enumeration of bacterial burdens in SCID mice infected with wild type *M. tuberculosis* H37Rv and the complementing strain (*panCD* in single copy integrated into the chromosome) showed a rapid increase in bacterial numbers in spleen, liver and lung before they succumbed to infection. In contrast, mice infected with Δ*panCD* mutant, showed an initial drop in bacterial numbers in spleen and liver followed by a

steady increase to reach 10^8 in the lungs at 160 days, at which time all mice were still alive (Fig. 6B).

Having demonstrated the significant attenuation of Δ*panCD* mutant, we sought to address the *in vivo* growth characteristics of this mutant in immune-competent BALB/c mice. All BALB/c mice infected with H37Rv succumbed to infection by day 25 with a MST of 22 days. Similarly, mice infected with the *panCD*-complemented strain were highly virulent with 100% mortality between 3-8 weeks post-infection similar to the wild type strain, with a MST of 28 days. In contrast, all mice infected with Δ*panCD* mutant survived for over 33 weeks demonstrating the severe attenuation phenotype of this mutant in immune-competent mice (Fig. 7A). Interestingly, bacterial enumeration at three weeks post infection showed 1 log increase in the Δ*panCD* numbers in lungs followed by a state of persistence with the onset of adaptive immune response. This growth characteristic was observed only in the lung but not in spleen or liver (Fig. 7B,C). A desirable trait of an effective live attenuated vaccine strain is its ability to grow within the host in a limited fashion in order to produce *in vivo* all the important protective antigens (McKenney, 1999; McKenny, 2000; Kanai, 1955). The Δ*panCD* mutant exhibits this characteristic in the lung, which is the primary site of infection in humans and does not get cleared over a prolonged period in all the three organs. The earlier auxotrophs of *M. tuberculosis* failed to grow in any of the organs and hence failed to adequately protect against experimental challenge in guinea pigs (Jackson, 1999), or mice.

The ability of the Δ*panCD* mutant to exhibit limited growth in the lung until the onset of adaptive immune response suggests that an unidentified putative pantothenate permease is able to transport this nutrient into resting macrophages, as in the SCID mice. A sodium-dependent pantothenate permease actively transports pantothenate into the cell of *Escherichia coli* (Vallari and Rock, 1985; Jackowski and Alix, 1990), *Plasmodium falciparum* (Saliba and Kirk, 2001) and mammals. Subsequent activation of macrophages leads to restricted supply of this nutrient within the phagosome resulting in growth arrest of the mutant. Pantothenic acid or its derivatives have been reported to confer resistance to radiation and oxidative stress by virtue of their role in biosynthesis of CoA and also by indirectly increasing the cellular supply of glutamate, a precursor of glutathione (Slyshenkov, 1995). Pantothenate kinase (*PanK*) mutants of Drosophila display membrane defects and improper mitosis and meiosis due to decreased phospholipid biosynthesis (Afshar et al., 2001). The disruption of *de novo* pantothenate biosynthesis causes an increased susceptibility of the Δ*panCD* mutant to reactive oxygen and nitrogen intermediates that are released within activated macrophages.

Having observed the Δ*nadBC* mutant to be non-attenuated in SCID mice, we chose to study the *in vivo* growth kinetics of this mutant in the more resistant C57BL/6 mice background. During the first three weeks of infection, the number of wild type and mutant bacteria recovered from all three organs showed little or no difference. Their numbers gradually increased in the lungs to reach 10^6. However, with the onset of adaptive immune response at three weeks, when the growth of bacteria in the lungs of mice infected with H37Rv became constant and tightly controlled, bacterial load in the lungs of mice infected with Δ*nadBC* mutant showed a constant tendency for clearance to reach more than 1.5 log drop in the bacterial numbers compared to mice infected with wild type strain (Fig. 8A). This difference was preserved up to 24 weeks following infection.

The reduced ability of the Δ*nadBC* mutant to sustain an infection was accompanied by attenuated virulence clearly seen from the survival experiment (Fig. 8C). While all mice infected with the wild type strain succumbed to infection between day 90 and 179 (MST 116 days) all mice infected with the Δ*nadBC* mutant (n=12) remain alive for a period of more than 8 months (Fig. 8C).

Our observation of the attenuation phenotype of Δ*nadBC* mutant became obvious only after the onset of immune response, suggesting that once the macrophages become activated, they restrict the amount of available NAD or NAD intermediates causing a restricted growth of the mutant strain. This would be in agreement with the recently reported observations that a significant part of antimicrobial function of the macrophages could be attributed to the IFN-γ promoted enhanced expression of indolamine 2-oxygenase (IDO), the inducible enzyme controlling L-tryptophan catabolic pathway causing an almost complete depletion of L-tryptophan pool. The enhanced catabolism of L-tryptophan leads to increased *de novo* biosynthesis of NAD needed to protect the cells from the free radicals formed as a result of macrophage activation. Recently, several studies have demonstrated the involvement of the tryptophan catabolism in the antimicrobial mechanisms of the activated macrophages. Induction of IDO was found responsible for the inhibition of intracellular growth of *Toxoplasma*, *Leishmania*, *Legionella* and *Chlamydia*. The restricted intracellular growth of Δ*nadBC* mutant could be explained with the very little amount of free NAD or NAD intermediates available within the activated macrophages.

Having established the safety and persistence of Δ*panCD* and Δ*nadBC* in immunocompetent mice, the protective efficacy of these mutants were evaluated using an aerosol challenge model with virulent *M. tuberculosis*, using the methods described in

Example 1. The aerosol route of infection was chosen, as this is the natural route of infection in humans. To assess the capacity of the auxotrophic vaccines to restrict growth of virulent *M. tuberculosis* in the organs of infected mice, bacterial numbers were enumerated one month post-infection in lung and spleen. See Table 3. In the unimmunized controls, bacterial numbers rose rapidly in the spleen and lungs, in contrast mice infected with a single dose of Δ*panCD* displayed significant reduction in bacterial numbers in the spleen and lung ($p<0.05$, in comparison to unimmunized controls). Mice given two doses of Δ*panCD* displayed a statistically significant reduction in the bacterial numbers to 4.5 log units in the lung ($p<0.01$) and 3.7 log units in the spleen ($p<0.05$). Mice vaccinated with BCG showed comparable reduction in bacterial burden in the lung and spleen to 3.3 log units and 4.7 log units respectively ($p<0.01$). Mice immunized with one or two doses of Δ*nadBC* mutant conferred statistically significant protection ($p<0.01$ in comparison to unimmunized group) that is comparable to the protection afforded by BCG vaccination. Interestingly, mice immunized with the Δ*nadBC* mutant showed no detectable CFUs in the spleen suggesting that the vaccination completely prevented the hematogenous spread of wild type *M. tuberculosis* following aerosol challenge.

Table 3.

A

Experimental Group	Lung CFUs ($\log_{10}$)	Spleen CFUs ($\log_{10}$)
Naive	4.05 ± 0.21	3.94 ± 0.21
Δ*nadBC* (1 x sc)	3.37 ± 0.40 **	<2 **
Δ*nadBC* (2 x sc)	3.6 ± 0.35 **	<2 **
BCG (1 x sc)	3.46 ± 0.19 **	<2 **

B

Experimental Group	Lung CFUs ($\log_{10}$)	Spleen CFUs ($\log_{10}$)
Naive	5.56 ± 0.05	4.35 ± 0.21
Δ*panCD* (1 x sc)	4.99 ± 0.17 (-0.57) *	3.65 ± 0.15 (-0.70)*
Δ*panCD* (2 x sc)	4.55 ± 0.09 (-1.01) **	3.73 ± 0.21 (-0.62)*
BCG (1 x sc)	4.71 ± 0.21 (-0.85) **	3.35 ± 0.20 (-1.00)**

p<0.05 compared to naïve, ** p<0.01 compared to naïve

Table 3. The attenuated *M. tuberculosis* Δ*nadBC* and Δ*panCD* mutants protect against aerogenic challenge with *M. tuberculosis* Erdman. Groups of C57BL/6 mice (5 mice per group) were vaccinated subcutaneously either once or twice (6 weeks apart) with 10^6 CFUs of mutant strains. Control mice were vaccinated subcutaneously with 10^6 CFUs of BCG-Pasteur. Three months after the initial immunization with either Δ*nadBC or* Δ*panCD* mutant or BCG, the mice were aerogenically challenged with approximately 100 CFUs of acriflavin-resistant *M. tuberculosis* Erdman (Ac^rMTB) strain as described earlier (Collins, 1985) After 28 days, the challenged mice were sacrificed, and the lungs and spleens of individual mice were removed aseptically and homogenized separately in 5 ml of Tween 80-saline using a Seward stomacher 80 blender (Tekmar, Cincinnati, OH). The homogenates were diluted serially in Tween 80 saline and plated on Middlebrook 7H11 agar with or without appropriate supplements as required. Samples from the BCG-vaccinated controls were plated on 7H11 agar containing 2 mg of thiophenecarboxylic acid hydrazide (Sigma Chemical Co., St Louis, MO) per ml to inhibit growth of any residual BCG. The CFU results were evaluated using the one-way ANOVA analysis of the Graph Pad InStat program. The numbers in paranthesis represent the differences between naïve and vaccinated organ CFUs.

In order to test the ability of the auxotrophic mutants to confer long lasting immunity, mice were challenged 7 months after an initial subcutaneous immunization with the Δ*nadBC* mutant. See Table 4. Mice immunized with Δ*nadBC* displayed significantly reduced numbers of the challenge organism in the lungs and no detectable numbers in the spleen comparable to the numbers seen in the BCG vaccinated mice. The results suggest that the Δ*nadBC* vaccine strain is able to persist within the mouse organs sufficiently long to mount a long lasting immunity to control subsequent infection.

Table 4.

Experimental Group	Lung CFUs (log_{10})	Spleen CFUs (log_{10})
Naive	4.61 ± 0.07	4.07 ± 0.20
BCG	4.00 ± 0.13*	2
NAD (1 x iv)	3.28 ± 0.15**	<2
NAD (2 x iv)	2.95 ± 0.14**	<2
NAD (1 x sc)	4.05 ± 0.12*	<2
NAD (2 x sc)	3.94 ± 0.13*	<2

*P<0.05; **P<0.01 by Dunnett's Multiple Comparison Test

Table 4. Immunizations with the Δ*nadBC* mutant confer long-term protection against an aerosol challenge. Groups of C57BL/6 mice (5 mice per group) were vaccinated subcutaneously or intravenously either once or twice (6 weeks apart) with 10^6 CFUs of Δ*nadBC* mutant. Control mice were vaccinated subcutaneously with 10^6 CFUs of BCG-Pasteur. Seven months after the initial immunization with either Δ*nadBC* mutant or BCG, the mice were aerogenically challenged with approximately 50 CFUs of acriflavin-resistant *M. tuberculosis* Erdman (Ac^rMTB) strain and the bacterial numbers at 28 days post challenge enumerated as described in Table 1.

To the best of our knowledge this is the first report of any *M. tuberculosis* auxotrophic vaccines administered subcutaneously to confer protection comparable to the conventional BCG vaccine strain in a mouse model of infection. Mice vaccinated with the Δ*panCD* and Δ*nadBC* survived for over one year following the aerosol challenge indicating the protection and safety of these vaccine strains.

Example 3. A pantothenate auxotroph of *Mycobacterium tuberculosis* is highly attenuated and protects mice against tuberculosis.

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Example summary

With the advent of HIV and the widespread emergence of drug resistant strains of *Mycobacterium tuberculosis*, newer control strategies in the form of a better vaccine could decrease the global incidence of tuberculosis. A desirable trait in an effective live attenuated vaccine strain is its ability to persist within the host in a limited fashion in order to produce important protective antigens in vivo (Kanai and Yanagisawa, 1955; McKenney et al., 1999). Rationally attenuated *M. tuberculosis* vaccine candidates have been constructed by deleting genes required for growth in mice (Jackson et al., 1999; Hondalus et al., 2000; Smith et al., 2001). These candidate vaccines failed to elicit adequate protective immunity in animal models, due to their inability to persist sufficiently long within the host tissues. Here we report that an auxotrophic mutant of *M. tuberculosis* defective in the *de novo* biosynthesis of

pantothenic acid (vitamin B5) is highly attenuated in immunocompromised SCID mice and in immunocompetent BALB/c mice. SCID mice infected with the pantothenate auxotroph survived significantly longer than mice infected with either BCG vaccine or virulent *M. tuberculosis* (250 days, vs. 77 days, vs. 35 days). Subcutaneous immunization with this auxotroph conferred protection in C57BL/6J mice against an aerosol challenge with virulent *M. tuberculosis*, which was comparable to that afforded by BCG vaccination. Our findings highlight the importance of de novo pantothenate biosynthesis in limiting the intracellular survival and pathogenesis of *M. tuberculosis* without reducing its immunogenicity in vaccinated mice.

Materials and Methods.

Media and Strains. *M. tuberculosis* H37Rv, *M. tuberculosis* Erdman and *M. bovis* BCG Pasteur were obtained from the Trudeau Culture Collection (Saranac Lake, NY) and cultured in Middlebrook 7H9 broth and 7H11 agar supplemented with 10% OADC, 0.5% glycerol, and 0.05% Tween 80. When required, pantothenate (24 μg/ml), hygromycin (50 μg/ml) or kanamycin (25 μg/ml) was added. Stock strains were grown in Middlebrook 7H9 broth in roller bottles and harvested in mid-logarithmic growth phase, before being stored in 1 ml vials at -70° C until required.

Disruption of panCD genes in M. tuberculosis. Specialized transduction was employed to disrupt the chromosomal copy of the *panCD* genes as described (U.S. Patent 6,271,034). Briefly, the 823 bp region upstream to the panC gene was amplified using primers Pan1 (5'-GTGCAGCGCCATCTCTCA-3')(SEQ ID NO:9) and Pan2 (5'-GTTCACCGGGATGGAACG-3')(SEQ ID NO:10). A 716 bp region downstream to the panD gene was amplified using primers Pan3 (5'-CCCGGCTCGGTGTGGGAT-3')(SEQ ID NO:11) and Pan4 (5'-GCGCGGTATGCCCGGTAG-3')(SEQ ID NO:12). PCR products were cloned with the TOPO TA cloning kit (Invitrogen, CA), and sequenced. PCR products were subsequently cloned into pJSC347, flanking a hygromycin cassette to create pSKV1. PacI digested pSKV1 was ligated into the temperature-sensitive mycobacteriophage phAE159 derived from TM4 and transduced as described earlier (Glickman et al., 2000; Raman et al., 2001). Genomic DNAs from hygromycin-resistant and pantothenate-requiring colonies were digested with *Bss*HII, and probed with a 716 bp downstream region, flanking the *M. tuberculosis panCD* operon to confirm the deletion. For complementation, the *M. tuberculosis panCD* operon was amplified by PCR from genomic DNA with its putative promoter, cloned

with TA cloning kit, sequenced, and subcloned into pMV306kan, a site-specific integrating mycobacterial vector.

Animal infections. C57BL/6, BALB/cJ and BALB/c SCID mice (6-8 weeks old) were purchased from Jackson Laboratories and were infected intravenously through the lateral tail vein. For time-to-death assays, BALB/c SCID mice were infected intravenously with $1x10^2$ CFU of *M. tuberculosis* H37Rv, $1x10^2$ CFU of *panCD*-complemented strain, $1x10^5$ CFU of Δ*panCD* mutant, or $1x10^5$ CFU of *M. bovis* BCG-P. For mouse organ CFU assays, BALB/cJ mice were infected with $1x10^6$ CFU of *M. tuberculosis* H37Rv or the *panCD*-complemented strain or the Δ*panCD* mutant. At appropriate time points, groups of 4-5 mice were sacrificed and the selected organs were homogenized separately in PBS/0.05% Tween 80, and colonies were enumerated on 7H11 plates grown at 37° C for 3-4 weeks (see McKinney et al., 2000). Pathological examination was performed on tissues fixed in 10% buffered formalin. The CFU results were evaluated using the one-way ANOVA analysis of the Graph Pad InStat program. All animals were maintained in accordance with protocols approved by the Albert Einstein College of Medicine Institutional Animal Care and Use Committee.

Vaccination Studies. Groups of C57BL/6 mice (5 mice per group) were vaccinated subcutaneously either once or twice (6 weeks apart) with $1x10^6$ CFU of the Δ*panCD* mutant strain. Control mice were vaccinated subcutaneously with $1x10^6$ CFU of *M. bovis* BCG-P. Three months after the initial immunization with either the Δ*panCD* mutant or BCG, the mice were aerogenically challenged with approximately 50-100 CFU of *M. tuberculosis* Erdman strain as described earlier. At 28 days following aerosol challenge, the challenged mice were sacrificed; the lungs and spleens of individual mice were removed aseptically and homogenized separately in 5 ml of Tween 80-saline using a Seward Stomacher 80 blender (Tekmar, Cincinnati, Ohio). The homogenates were diluted serially in Tween 80 saline and plated on Middlebrook 7H11 agar with or without appropriate supplements as required. Samples from the BCG-vaccinated controls were plated on 7H11 agar containing 2 mg/ml of thiophene-2-carboxylic acid hydrazide (Sigma) to inhibit growth of any residual BCG.

Results and Discussion.

Lipid biosynthesis and metabolism have been shown to play a pivotal role in the intracellular replication and persistence of *M. tuberculosis* (Cox et al., 1999; Camacho et al., 1999; Glickman et al., 2000; De Voss et al., 2000; Manca et al., 2001; McKinney et al., 2000). Therefore, we sought to globally impair the ability of this bacterium to synthesize lipids. Pantothenic acid (vitamin B5) is an essential molecule required for the synthesis of coenzyme

A (CoA) and acyl carrier protein (ACP), that play important roles as acyl group carriers in fatty acid metabolism, the tricarboxylic acid cycle, biosynthesis of polyketides and several other reactions associated with intermediary metabolism (Jackowski, 1996). Bacteria, plants and fungi synthesize pantothenate from amino acid intermediates, whereas it is a nutritional requirement in higher animals (FIG 9a).

We constructed a double deletion mutant of *M. tuberculosis* in the *panC* and *panD* genes that are involved in the de novo biosynthesis of pantothenate (FIG. 9b,c). The Δ*panCD* mutant was found to be auxotrophic for pantothenate with no detectable reversion to prototrophy when $1x10^{10}$ cells were plated on minimal medium. The growth rate of the mutant was identical to wild type H37Rv in broth cultures in the presence of exogenous pantothenate (data not shown). The attenuation of the Δ*panCD* mutant was assessed by infection of immunocompromised SCID mice. SCID mice infected intravenously with H37Rv succumbed to the resulting infection in about 5 weeks. In contrast, all mice infected with the Δ*panCD* mutant survived for more than 36 weeks (average, 253 days) (FIG. 10a). This attenuation is due to pantothenate auxotrophy as the full virulence phenotype was restored when the *panCD* wild type genes were integrated into the chromosome of the Δ*panCD* mutant in single copy. Enumeration of bacterial burdens in SCID mice infected with H37Rv and the Δ*panCD*-complemented strain showed a rapid increase in bacterial numbers in the spleen, liver and lung, until they succumbed to infection. In contrast, mice infected with the Δ*panCD* mutant showed an initial drop in bacterial numbers in the spleen and liver followed by a gradual increase in the number of viable bacteria, reaching $1x10^6$ colony-forming units (CFU) by day 224 (FIG. 10b). Notably, the CFU values increased to $1x10^8$ in the lungs of the infected mice. The ability of Δ*panCD*-infected SCID mice to survive despite a substantial bacterial burden in their lungs emphasizes the extent of attenuation in this mutant and compares with the phenotype observed with the *M. tuberculosis whiB3* and *sigH* mutants described recently (Steyn et al., 2000; Kaushal, 2000). Notably, SCID mice infected with bacille Calmette-Guerin-Pasteur (BCG-P) strain succumbed to infection by 83 days (Weber et al., 2000) in contrast to the prolonged survival observed in Δ*panCD*-infected mice.

Studies in immunocompetent mice further demonstrate the attenuation of the Δ*panCD* mutant. Survival studies showed that BALB/c mice infected with H37Rv succumbed to infection by day 25 (average, 21 days) and mice infected with an identical dose of the *panCD*-complemented strain succumbed to infection between days 21 to 53 (average, 37 days). Importantly, all mice infected with $1x10^6$ CFU of the Δ*panCD* mutant survived 375 days, when

the experiment was terminated (FIG. 10c). At 3 weeks post infection, in contrast to the H37Rv strain, BALB/c mice infected with Δ*panCD* mutant showed a 10-fold increase in bacterial numbers in the lungs followed by a gradual decline in viable numbers over the next 38 weeks of infection (FIG. 10d) and the bacterial burden gradually declined in the spleen and liver throughout the course of infection (FIG. 10e). Histopathologic examination of the lungs from mice infected with either H37Rv or the Δ*panCD*-complemented strain, showed severe, diffuse lobar granulomatous pneumonia (FIG. 11a,b). The pneumonia affected more than 50% of the lung, and was pyogranulomatous with marked necrosis in the advanced consolidated areas, particularly in the lungs of mice challenged with H37Rv. Both of these strains caused severe granulomatous splenitis and widespread granulomatous hepatitis. At 3 weeks post-infection with the Δ*panCD* mutant, low to moderate numbers of focal infiltrates of mononuclear cells scattered throughout the lung were seen (FIG. 11c). The spleen was moderately enlarged with scattered granulomas. Similarly, the liver showed numerous focal granulomas. At 24 weeks post-infection, consistent with the bacterial numbers, histological examination of the lungs from mice infected with the Δ*panCD* mutant showed only occasional focal mild infiltrations, predominately lymphocytic (FIG. 11d). The spleen showed only mild histiocytic hyperplasia and there were fewer, focal, predominately lymphocytic accumulations in the liver.

The mechanisms that allow the persistence of the Δ*panCD* mutant bacteria for over 8 months in the SCID mouse model remain unclear. We speculate the functional role of an unidentified permease in transporting adequate amount of pantothenate in the Δ*panCD* mutant that allows its persistence but not the ability to cause disease. A pantothenate permease that transports pantothenate have been described in *Plasmodium falciparum* and *Escherichia coli* (Saliba and Kirk, 2001; Jackowski and Alix, 1990). In the lungs of immunocompetent mice, an initial growth of the Δ*panCD* mutant during the first 3 weeks is followed by a steady decline in bacterial numbers following the onset of an adaptive immune response. The intracellular lifestyle of *M. tuberculosis* poses significant challenges to the bacterium in acquiring essential nutrients. Pantothenic acid or its derivatives have been shown to confer resistance to oxidative stress (Slyshenkov et al., 1996) and lack of pantothenate biosynthesis in the Δ*panCD* mutant may render it more susceptible to such adverse effects. Likewise, a pantothenate kinase (*panK*) mutant of *Drosophila* was shown to display membrane defects and improper mitosis and meiosis due to decreased phospholipid biosynthesis (Afshar et al., 2001). Therefore, it is plausible that the pantothenate salvage pathway is inadequate in restoring full virulence of the Δ*panCD* mutant in the absence of a functional de novo biosynthetic pathway.

As a test of vaccine potential, immunized mice were challenged with virulent *M. tuberculosis* Erdman by the aerosol route (Collins, 1985). Following subcutaneous immunization, the Δ*panCD* mutant could not be detected in the spleens or lungs of mice at 8 and 12 weeks. In the naive controls, the bacterial CFU values increased 10,000-fold in the lung during the first month after challenge. Similarly, substantial dissemination and growth in the spleen was detected within one month of the challenge in naive controls. In contrast, mice immunized with single or double doses of the Δ*panCD* mutant displayed statistically significant reductions ($P < 0.05$) in lung and spleen CFU values relative to naive controls. Mice vaccinated with BCG showed similar reduction in organ bacterial burdens compared to the nonimmunized controls (FIG. 11e,f). In these aerogenic challenge studies, no significant differences were detected in the lung and spleen CFU values for mice vaccinated with either the Δ*panCD* mutant strain or with BCG. At 28 days after the aerogenic challenge with virulent *M. tuberculosis*, histopathological examination of lungs of Δ*panCD* immunized mice revealed a less severe infection relative to the unvaccinated control mice. In controls, severe bronchitis, moderate pneumonia, and spread of the infection to the adjacent lung parenchyma was observed. By comparison, the Δ*panCD* vaccinated mice had milder bronchitis and smaller areas of mild interstitial pneumonitis, with localized areas of granulomatous pneumonia in some mice. Importantly, no lung pathology was detected in vaccinated mice at the time of challenge (data not shown). Two groups of mice that were vaccinated with one or two doses of the Δ*panCD* mutant and then challenged with *M. tuberculosis* Erdman were active and healthy for more than one year following the virulent challenge. Histopathological analysis of lung sections from these mice showed only mild inflammation and fibrosis despite the chronic infection.

By creating a *M. tuberculosis* strain that is defective in pantothenate biosynthesis, we have taken a critical step in the rational development of an attenuated *M. tuberculosis* vaccine strain. We have shown that a functional pantothenate biosynthetic pathway, which is required for the synthesis of complex mycobacterial lipids, is essential for the virulence of *M. tuberculosis*. Although the precise mechanism of the reduced virulence is unclear, it is reasonable to speculate that this could be due to reduced synthesis of toxic polyketides and secreted lipids or a general slow down of metabolism. Tubercle bacilli lacking the two genes required to synthesize pantothenate failed to revert and were highly attenuated and less virulent than BCG vaccine when tested in the rigorous SCID mouse model of infection. Despite the reduced virulence associated with the deletion of the *panCD* genes, these vitamin auxotrophs

remain persistent *in vivo* as shown by their ability to survive for at least eight months in immunocompetent mice. The persistence of this mutant strain undoubtedly contributes to the substantial immunogenicity seen in the mouse tuberculous challenge model. Overall, the Δ*panCD* mutant has many of the characteristics necessary for a live vaccine candidate strain: it is attenuated by a non-reverting mutation and essentially avirulent while being persistent and immunogenic. Given the genetic differences between *M. bovis* and *M. tuberculosis* (Behr et al., 1999), one would predict that a rationally attenuated *M. tuberculosis* strain would have a more relevant repertoire of species-specific antigens and thus should elicit, in humans, more effective protective immune responses against tuberculous infections than BCG.

Example 4. The primary mechanism of attenuation of BCG is a loss of invasiveness due to host cell lysis.

Example Summary.

Tuberculosis remains a leading cause of death worldwide, despite the availability of effective chemotherapy and a vaccine. BCG, the tuberculosis vaccine, is an attenuated mutant of *M. bovis* that was isolated following serial subcultivations, yet the basis for this attenuation has never been elucidated. A single region (RD1), deleted in all BCG substrains, was deleted from virulent *M. bovis* and *M. tuberculosis* strains and the resulting three Δ*RD1* mutants were significantly attenuated for virulence in both immunocompromised and immunocompetent mice. Like BCG, *M. tuberculosis* Δ*RD1* mutants protect mice against aerosolized *M. tuberculosis* challenge and these mutants also consistently display altered colonial morphotypes. Interestingly, the Δ*RD1* mutants failed to cause necrosis, via lysis, of pneumocytes, a phenotype that had been previously used to distinguish virulent *M. tuberculosis* from BCG. We conclude that the primary attenuating mechanism of BCG is the loss of cytolytic activity, resulting in reduced invasiveness.

Introduction.

BCG (bacille Calmette and Guerin), was first isolated from *M. bovis* following serial subculturing of *M. bovis* in 1908 (Calmette and Guerin, 1909). Drs. Calmette and Guerin set out to test the hypothesis that a bovine tubercle bacillus could transmit pulmonary tuberculosis following oral administration (Calmette and Guerin, 1905; Gheorghiu, 1996) and developed a medium containing beef bile that enabled the preparation of fine homogenous bacillary suspensions. After the 39th passage, the strain was found to be unable to kill experimental animals (Calmette and Guerin, 1909). Between 1908 and 1921, the strain showed no reversion

to virulence after 230 passages on bile potato medium (Gheorghiu, 1996), which is consistent with the attenuating mutation being a deletion mutation. In the animal studies that followed, BCG was shown to be attenuated, but it also protected animals receiving a lethal challenge of virulent tubercle bacilli (Calmette and Guerin, 1920). BCG was first used as a vaccine against tuberculosis in a child in 1921 (Weill-Halle and Turpin, 1925). From 1921 to 1927, BCG was shown to have protective efficacy against TB in a study on children (Id.; Calmette and Plotz, 1929) and was adopted by the League of Nations in 1928 for widespread use in the prevention of tuberculosis. By the 1950's, after a series of clinical trials, the WHO was encouraging widespread use of BCG vaccine throughout the world (Fine and Rodrigues, 1990). Although an estimated 3 billion doses have been used to vaccinate the human population against tuberculosis; the mechanism that causes BCG's attenuation remains unknown.

Mahairas et al. (1996) first compared the genomic sequences of BCG and *M. bovis* using subtractive hybridization and found that there were three Regions of Difference (designated *RD1*, *RD2*, and *RD3*) present in the genome of *M. bovis*, but missing in BCG. Behr et al. (Behr et al., 1999) and others (Gordon et al., 2001) later identified 16 large deletions, including *RD1* to *RD3*, present in the BCG genome but absent in *M. tuberculosis*. Eleven of these 16 deletions were unique to *M. bovis*, while the remaining 5 deletions were unique to BCG. One of these 5 deletions, designated *RD1* (9454 bp), was absent from all of the BCG substrains currently used as TB vaccines worldwide and it was concluded that the deletion of *RD1* appeared to have occurred very early during the development of BCG, probably prior to 1921 (Behr et al., 1999). It is reasonable to hypothesize that *RD1* was the primary attenuating mutation first isolated by Calmette and Guerin to generate BCG from *M. bovis*. Attempts to restore virulence to BCG with *RD1*-complementing clones have been unsuccessful (Mahairas et al., 1996).

<u>Results.</u>

<u>*RD1* deletions of *M. bovis* and *M. tuberculosis* are attenuated for virulence in immunocompromised mice.</u> To test if *RD1* is essential for virulence in *M. bovis* and *M. tuberculosis*, it was necessary to delete the *RD1* (FIG. 1a) from virulent strains, demonstrate loss of virulence, and then restore virulence by complementation with the *RD1* DNA. Since the original *M. bovis* parent of BCG was lost in World War I (Grange et al., 1983), we initiated studies with virulent *M. bovis* Ravenel and a variety of virulent *M. tuberculosis* strains. Despite success in generating ~~an unmarked~~ deletion mutant of *RD1* in *M. tuberculosis* with a plasmid transformation system[1,2], over 100 independent transformations failed to yield an *RD1*

deletion in *M. bovis*. As an alternative strategy, specialized transduction (Bardarov et al., 2002)[3] was successfully used to generate *RD1* deletion mutants not only in *M. bovis* Ravenel, but also the H37Rv, Erdman, and CDC1551 strains of *M. tuberculosis* (FIG. 12). This deletion represents the largest deletion mutation generated by a targeted disruption in *M. tuberculosis* or *M. bovis* made to date and demonstrates the utility of the specialized transduction system. Moreover, since the parental specialized transducing phage has been shown to infect over 500 clinical *M. tuberculosis* isolates (Jacobs et al., 1987), it should be possible to introduce the *RD1* deletion into any *M. tuberculosis* or *M. bovis* isolate of interest.

To determine if the *RD1* deletion causes an attenuating phenotype in *M. bovis* and *M. tuberculosis*, the *M. tuberculosis* H37Rv Δ*RD1* was inoculated intravenously into immunocompromised mice possessing the SCID (severe combined immunodeficiency) mutation. Groups of ten mice were injected intravenously with either 2×10^6 wild type or Δ*RD1* strain of *M. tuberculosis* and *M. bovis*, and three mice per group were sacrificed 24 hours later to verify the inoculation doses. All of the SCID mice infected with the parental *M. tuberculosis* or *M. bovis* strain died within 14 to 16 days post-infection (FIG. 12A). In contrast, the SCID mice infected with equal doses of the Δ*RD1* strains of *M. tuberculosis* or *M. bovis* were all alive at 25 to 41 days post-infection, demonstrating a highly significant attenuation of the virulence of both strains. It is important to note that BCG-Pasteur kills SCID mice approximately 70 days post-infection (FIG. 13B), suggesting that BCG substrains have acquired additional attenuating mutations which are consistent with the deletion analysis of BCG strains (Behr et al., 1999) and the previous failures to restore virulence with the RD1 region (Mahairas et al., 1996).

To prove that the attenuation of virulence was due to the *RD1* deletion, the *M. tuberculosis* Δ*RD1* was transformed with an integrating cosmid, 2F9, containing the *RD1* region from *M. tuberculosis* H37Rv[4]. SCID mice were infected as described above and the attenuation for virulence was restored to the parental virulent phenotype (FIG. 13B). These results strongly suggest that the genes deleted from the *RD1* region contribute to the virulence phenotype.

<u>The M. tuberculosis Δ*RD1* is highly attenuated in immunocompetent BALB/c mice.</u> The virulence of the *M. tuberculosis* Δ*RD1* mutant was further assessed by intravenous inoculation of immunocompetent BALB/c mice. While the virulent parent *M. tuberculosis* strain killed the BALB/c mice in 10 to 17 weeks post-infections, 100% of mice were alive at 48 weeks and 43 weeks post-infections in two independent experiments (FIG. 13C).

While infection with BCG and *M. tuberculosis* Δ*RD1* yielded similar survival results in BALB/c mice, there were substantial differences in the growth kinetics in mice. BCG grew in a limited fashion in lungs, liver and spleen in BALB/c mice during the 22 weeks of the experiment (FIG. 4B-D). In contrast, the *M. tuberculosis* Δ*RD1* strain grew in a fashion indistinguishable from the parental *M. tuberculosis* H37Rv in all mouse organs for the first 8 weeks. Thereafter, mice infected with the parental *M. tuberculosis* failed to contain the infection leading to mortality. Strikingly, mice infected with the *M. tuberculosis* Δ*RD1* showed a definite control over infection resulting in significantly prolonged survival of mice (FIG. 4B-D).

Histopathological examination further demonstrated that the mutant was attenuated in virulence compared to the parent strain H37Rv (FIG. 5D-F). In contrast to the rapidly progressive infection with the parent strain, the lung lesions caused by the mutant were maximal in mice examined at 8 weeks post-infection. Consolidating granulomatous pneumonia involved an estimated 25-30% of the lung in these mice. Numerous organisms were demonstrated by acid fast staining. The pneumonia subsequently underwent partial resolution. By 14 weeks, and again, at 22 weeks post-infection, the lungs showed peribronchial and perivascular inflammatory cell accumulations and focal, generally non-confluent, granulomas now with a prominent lymphocytes infiltration. The numbers of acid fast bacilli were reduced. Liver lesions consisted of low numbers of scattered granulomas. Spleens were smaller, with persistent granulomas in the red pulp. Mice infected with *M. bovis* BCG showed mild lesions in the lung, liver and spleen at all time points (FIG. 5G-I). At longer time intervals post-infection the lesions were fewer in number, and smaller with prominent lymphocytic infiltrations. At 14 weeks post-infection, *M. bovis* BCG was below the level of detection by acid fast staining. In summary, whereas *M. tuberculosis* Δ*RD1* initially grew in a manner similar to the parental *M. tuberculosis* H37Rv, this *RD1* mutant was limited in the extent of spread of infection, particularly in the lung. This contrasts the extensive and severe damage caused by the parent strain. The subsequent resolving granulomas, localization of the lesions and changes in the composition of the inflammatory cell infiltrations suggested that the mice mounted an effective immune response to combat *M. tuberculosis* Δ*RD1* infection and thereby reduced the numbers of viable organisms.

<u>Early BCG properties: Altered colonial morphotypes and long-term immunogenicity.</u> While frozen stocks of the original BCG strain do not exist, written records do exist describing the early BCG strains, as Dr. Calmette sent the strains to many laboratories. In a study

published in 1929, Petroff and colleagues reported that BCG displayed two distinct colony types (Petroff et al., 1929). One morphotype was a smooth (S) phenotype that was flat and corded (like the parental virulent strain) and the second was a rough and raised (R) phenotype. The *M. tuberculosis* Δ*RD1* mutant was generated independently four times and consistently yielded a 20 to 50% mixture of two colonial morphotypes on Middlebrook medium without Tween 80 (FIG. 3B). The distinction of these two types of morphology could be noted even when the colonies were less than two weeks old, as the rough colonies were constricted and elevated with only a small portion of the base of the colony attached to the agar, while the smooth colonies tended to be flattened and spread out. When colonies grew older, e.g. 6 weeks old, the rough colonies remained more constricted compared to those of smooth colonies. The rough colonies exhibited large folds on the surface (FIG. 3F-G), as compared to those of the smooth colonies that exhibited small wrinkles (FIG. 3E).

The generation of two distinct colonial morphotypes must be a phenotypic change induced by the deletion of *RD1*. The morphotypes could not be cloned, as repeated subculturing of either the R or S colonies continued to yield both colonial morphotypes. Moreover, Southern analysis of R and S colonies revealed each morphotype had the same *RD1*-deleted genotype (FIG. 3D). Furthermore, complementation of *M. tuberculosis* Δ*RD1* with the *RD1* region restored the mutant phenotype back to the homogenous parental S phenotype (FIG. 3A-C). These results suggest that the variable morphotypes resulted directly from the *RD1* deletion. It can therefore be postulated that a regulator of colonial morphology is affected by one or more of the deleted genes.

One of the hallmark characteristics of BCG is its ability to provide protection against aerosolized challenge with virulent *M. tuberculosis*. To test the potential of *M. tuberculosis* Δ*RD1* to immunize and protect mice against tuberculous challenge, we used the model of subcutaneous immunization of C57BL/6 mice followed by an aerogenic challenge with virulent *M. tuberculosis* (McGuire et al., 2002). Groups of mice were vaccinated subcutaneously with either $1x10^6$ BCG 9 or $1x10^6$ *M. tuberculosis* Δ*RD1*. Eight months following vaccination, the mice were all healthy, thereby demonstrating attenuation in a third mouse strain. Vaccinated and unvaccinated mice were aerogenically challenged with 200 CFU of the acriflavin-resistant strain of *M. tuberculosis* Erdman. Twenty-eight days after the challenge, the mice were sacrificed and the bacterial burden in the lungs and spleens were determined (see Table 5). Naive mice served as controls. While the acriflavin-resistant *M. tuberculosis* grew to 6.61±0.13 (log^{10} CFU) in lungs of naive mice, both the BCG-vaccinated

and *M. tuberculosis* Δ*RD1*-vaccinated mice exhibited greater than 1 log protection in lungs with CFU values of 5.07±0.10 ($p < 0.001$ relative to controls) and 5.11±0.14 ($p < 0.001$), respectively, detected at the four week time point. The *M. tuberculosis* Δ*RD1* also protected against hematogenous spread; CFU values in the spleen were 5.26±0.11 for the controls, 4.00 ±0.33 ($p < 0.01$) for the *M. tuberculosis* Δ*RD1* immunized mice, and 3.85±0.17($p < 0.01$) for the BCG vaccinated animals. Thus, the *M. tuberculosis* Δ*RD1* shares long-term immunogenicity like BCG.

Table 5. Bacterial burden of virulent *M. tuberculosis* in uninoculated mice and mice inoculated with BCG and H37Rv Δ*RD1*.

Vaccination strain	Lung (log_{10}CFU)	Spleen (log10CFU)
-	6.61 ± 0.13	5.26 ± 0.11
BCG	5.07 ± 0.10***	3.85 ± 0.17**
H37Rv Δ*RD1*	5.11 ± 0.14***	4.00 ± 0.33**

** p<0.01; *** p<0.001.

Discussion

BCG is a mutant of *M. bovis* that was isolated over 94 years ago and characterized for its attenuation for virulence in animals. For over 80 years, BCG has been used as a tuberculosis vaccine having been given to 3 billion humans. It is currently the only anti-tuberculous vaccine available for use in humans, yet its precise attenuating mutations and mechanisms of attenuation have never been determined. Previous studies had identified regions of the *M. bovis* chromosome that were absent from BCG, but present in virulent *M. bovis* and *M. tuberculosis* strains (Mahairas et al., 1996; Gordon et al., 2001). An elegant microarray analysis has also demonstrated that there was only one deletion common to all BCG strains; the authors hypothesized this was the primary attenuating mutation in the original BCG strain isolated by Drs. Calmette and Guerin (Behr et al., 1999).

Using a combination of targeted deletion mutagenesis, virulence assays, and complementation analysis, we have been able to unambiguously prove that *RD1* is required for virulence for *M. tuberculosis*, and by analogy for *M. bovis*, for the first time. Moreover, the combination of phenotypes associated with the early BCG strains: i) the attenuation for virulence, ii) the altered colonial morphotypes, and iii) the ability to confer

long-term immunogenicity in animals allow us to conclude that the RD1 deletion was the primary attenuating mutation in the original BCG isolate.

With regards to the Δ*RD1* mutant histology, at 22 weeks post infection, it was noted that the mutant was limited in the extent of the spread of infection, in contrast to the extensive damage caused by the parental strain. Interestingly, Pethe et al. (2001) determined that *M. tuberculosis* needs to bind and/or invade epithelial cells in order to disseminate and cause widespread destruction of the lung, whilst another study reported that pulmonary M cells can act as a portal of entry to the lung for the tubercle bacilli (Teitelbaum, 1999). In relation to *in vitro* analyses, studies utilizing a model of the alveolar barrier, consisting of pneumocytes and monocytes, described how *M. tuberculosis* infection of the pneumocytes resulted in cytolysis, which disrupted the barrier and allowed more efficient translocation of intracellular bacilli (Bermudez et al., 2002).

Notes

[1]The following four primers were used to amplify upstream and downstream flanking sequences (UFS and DFS, respectively) for the construction of the *RD1* deletion mutants. UFS was amplified using TH201: GGGGGCGCACCTCAAACC (SEQ ID NO:5) and TH202: ATGTGCCAATCGTCGACCAGAA (SEQ ID NO:6). DFS was amplified using TH203: CACCCAGCCGCCCGGAT (SEQ ID NO:7), and TH204: TTCCTGATGCCGCCGTCTGA (SEQ ID NO:8). Recognition sequences for different restriction enzymes were included at the ends of each primer to enable easier manipulation.

[2]The unmarked deletion mutant of *M. tuberculosis* H37Rv, mc^{2}4002, was generated by transformation using a *sacB* counterselection (Snapper et al., 1988; Pelicic et al., 1996; Pavelka et al., 1999). Specifically, the plasmid pJH508 was created by first cloning UFS into *Kpn*I and *Xba*I sites, then cloning DFS into *EcoR*I and *Hind*III sites of pJH12, a pMV261-derived *E. coli* - *Mycobacteria* shuttle plasmid, to create pJH506 in which UFS and DFS flanked a green fluorescent protein gene (GFPuv, Clonetech) whose expression was driven by the *M. leprae* 18Kd promoter. The UFS-gfp-DFS cassette was sub-cloned into the *EcoR*V site of plasmid pYUB657 to create pJH508. The first homologous recombination involved the identification of hygromycin resistant colonies, resulting from the transformation of *M. tuberculosis* with pJH508. Southern analysis of the *Nco*I-digested DNA isolated from hygromycin resistant colonies probed with UFS or DFS, confirmed the presence of a single copy of pJH508 inserted into the *M. tuberculosis* genome. The transformant (mc^{2}4000) identified was then grown in 7H9 broth to saturation, to allow the second homologous

recombination to occur, resulting in recombinants that could be selected by plating the culture on 7H10 plates, supplemented with 3% sucrose. Both Southern analysis and PCR of the DNA isolated from sucrose resistant colonies confirmed the RD1 deletion.

[3]Specialized transduction is a mycobacteriophage-based method for the delivery of homologous DNA constructs using conditionally replicating shuttle phasmids (Jacobs et al., 1987; Bardarov et al., 1997; Carriere et al., 1997) has been used successfully for *M. tuberculosis* (Glickman et al., 2000, 2001; Raman et al., 2001). Specifically, a transducing phage phAEKO1 was constructed by inserting UFS and DFS into pJSC347, flanking a hygromycin cassette, to create pJH313. pJH313 was digested with PacI and ligated to phAE159, a temperature-sensitive mycobacteriophage derived from TM4. The transduction was performed by growing *M. tuberculosis* to an $O.D._{600}$ of 1.0, washing twice with MP buffer (50 mM Tris pH 7.6, 150 mM NaCl, 10 mM $MgCL_2$, 2 mM $CaCl_2$), resuspending into an equal volume of MP buffer and mixing with the transducing phage phAEKO1 at an MOI of 10. The mixtures were incubated at 37°C overnight, then plated on 7H10 plates supplemented with hygromycin at 50 μg/ml. Hygromycin resistant colonies were analyzed by PCR and Southern analysis, as described above, to confirm the deletion of *RD1*.

[4]Complementation analyses was performed using the integration proficient cosmids (Skjot et al., 2000; van Pinxteren et al., 2000a) pYUB412 made by S. Bardarov, a library made by F. Bange, and cosmid identified and generously provided by S.T. Cole.

Example 5. Vaccine efficacy of a lysine auxotroph of *M. tuberculosis*

In this Example, we describe the *in vivo* growth phenotype and vaccine efficacy of a lysine auxotrophic mutant of *Mycobacterium tuberculosis* strain H37Rv. An immunization experiment using the mouse model with an aerosol challenge showed that two doses of the *M. tuberculosis* mutant were required to generate protection equivalent to that of the BCG vaccine.

Despite the existence of anti-microbial drugs and a widely used vaccine, *Mycobacterium tuberculosis* remains the primary cause of adult death due to a bacterial agent (Dolin et al., 1994). The emergence of multi-drug resistant strains of *M. tuberculosis*, the variable efficacy of the current vaccine, the bacille-Calmette and Geurin (BCG), and the HIV pandemic have all contributed to a growing global tuberculosis problem.

Several studies have described the development of attenuated auxotrophic strains of BCG and/or *M. tuberculosis* (Guleria et al., 1996); Hondalus et al., 2000; Jackson et al., 1999;

Smith et al., 2001). All of these studies utilized single immunization protocols and demonstrated differences in the protective responses thus elicited. In this study, we describe the *in vivo* growth characteristics of a previously described lysine auxotroph of *M. tuberculosis* H37Rv (Pavelka and Jacobs, 1999), and evaluate the vaccine potential of this mutant by a multiple immunization protocol in a mouse model of the human disease, using an aerosol challenge.

<u>Clearance of the *M. tuberculosis* lysine auxotroph in SCID mice.</u> Female SCID mice were bred at the animal facility of the Albert Einstein College of Medicine. The animals were maintained under barrier conditions and fed sterilized commercial mouse chow and water ad libitum. The *M. tuberculosis* strains Erdman, $mc^2 3026$ (Δ*lysA::res*) (Id.), and $mc^2 3026$ bearing pYUB651 (expressing the wild-type *lysA* gene) were grown in Middlebrook 7H9 broth (Difco) supplemented with 0.05% Tween-80, O.2% glycerol, 1X ADS (0.5% bovine serum albumin, fraction V (Roche); 0.2% dextrose; and 0.85% NaCl) or on Middlebrook 7H10 or 7H11 solid medium (Difco) supplemented with 0.2% glycerol and 10% OADC (Becton Dickinson). Culture media for the lysine auxotroph were supplemented with 1 mg/ml of L-lysine (for both liquid and solid media), and 0.05% Tween-80 was also added to solid medium. Liquid cultures were grown in 490 cm^2 roller bottles (Corning) at 4-6 rpm. Plates were incubated for 3-6 weeks in plate cans. All cultures were incubated at 37°C.

Titered frozen stocks of the bacteria were thawed and diluted appropriately in phosphate buffered saline containing 0.05% Tween-80 (PBST). The bacterial suspensions were plated at the time of injection to confirm the number of viable bacteria. Intravenous injections were given via a lateral tail vein. At various time points post-injection (24 hours, then once weekly), 3 mice were sacrificed, and the lungs, liver, and spleen removed and homogenized separately in PBST using a Stomacher 80 (Tekmar, Cincinnati, OH). The homogenates were diluted in PBST and plated to determine the number of CFU/organ. Note that mice were sacrificed at 24 hours post-injection in order to compare the bacterial colony forming units recovered from the mice with the colony forming units in the suspensions at the time of injection. Thus the bacterial counts reported at time zero actually represent the viable bacteria recovered from the mice at 24-hours post-injection.

The lysine auxotrophic strain was cleared from and did not appear to grow in the examined organs of the SCID mice, while the complemented strain multiplied extensively (FIG. 14). Interestingly, the auxotrophic inoculum was cleared from the spleens and lungs but persisted somewhat longer in the liver (FIG. 14B). The mice receiving the complemented *M.*

tuberculosis mutant died within three weeks of challenge, while the mice given the auxotrophic *M. tuberculosis* mutant did not display any gross organ pathology and survived for at least the duration of the experiment.

Two immunizations with the *M. tuberculosis* lysine auxotroph $mc^2 3026$ are required to match the efficacy of vaccination with BCG-Pasteur. We tested the vaccine potential of the lysine auxotroph $mc^2 3026$ in the mouse model by means of a virulent aerosol challenge. Female, pathogen-free C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were vaccinated intravenously with ca. 1×10^6 CFU of the *M. tuberculosis* lysine auxotroph or BCG-Pasteur suspended in 0.2 ml PBST. Mice vaccinated with $mc^2 3026$ were revaccinated at 4 week intervals and the number of viable organisms in the lungs and spleens determined weekly throughout the vaccination period, as described above for the SCID mouse experiments. Five mice were examined at each time point.

Immunized mice were challenged 3 months after the initial vaccination. A frozen aliquot of a *M. tuberculosis* Erdman stock was thawed and diluted in PBST to ca. 1×10^6 CFU/ml and 10 ml was introduced into the nebulizer of a Middlebrook aerosol chamber (Glas-Col, Terre Haute, IN). The mice were exposed to the infectious aerosol for 30 minutes, inhaling 50-100 CFU into their lungs over this period. Five mice were sacrificed immediately following the challenge period and the lung homogenates were plated to check the amount of the challenge inoculum actually reaching the lungs. Groups of vaccinated and control mice were sacrificed 14, 28, and 42 days later and the lung and spleen homogenates plated to determine the number of viable colony forming units of *M. tuberculosis* Erdman present. Data were analyzed using the Student's t-test and an analysis of variance between several independent means, using the In Stat Statistics program (GraphPad Software, San Diego).

A preliminary experiment demonstrated that a single intravenous immunization of immunocompetent C57BL/6 mice with the *M. tuberculosis* mutant did not generate a significant protective response to the subsequent aerosol challenge with virulent *M. tuberculosis* Erdman. In that experiment, the M. tuberculosis auxotroph was rapidly cleared from the mice (FIG. 15A), and the single immunization with the auxotroph was insufficient to reduce the bacterial burden in the lungs and spleens relative to a single immunization with BCG (FIG. 15B).

The failure of the auxotroph to confer protection might have been due to the inability of the mutant to persist long enough, or to synthesize enough antigen to induce an immune response that could significantly restrict the growth of the challenge organisms. One way to

circumvent this problem is to give multiple doses of vaccine (Collins, 1991; Homchampa et al., 1992). To this end, mice were intravenously immunized two or three times at four-week intervals with the *M. tuberculosis* lysine auxotroph. In both cases, the vaccine strain was cleared from the lungs and spleens of all the mice at rates similar to that seen with the single immunization experiment (FIG. 15A). Three months after the first immunization the mice were challenged with *M. tuberculosis* Erdman by the aerosol route and the bacterial counts in the lungs and spleens were determined and compared to a BCG-Pasteur immunized control, as well as the sham immunized controls. As seen in FIG. 15C, double immunization with the *M. tuberculosis* lysine auxotroph induced a protective response that was equivalent to that of the BCG control. The reduction in counts in the lung and spleen was equivalent to a 100-fold reduction in bacterial counts compared to the unvaccinated control (FIG. 15C). The results from the triple immunization experiment were essentially similar as those from the double immunization experiment described above (data not shown). Furthermore, mice that were immunized with three doses of the *M. tuberculosis* lysine auxotroph and challenged with virulent *M. tuberculosis* Erdman survived at least as long as the BCG-immunized control mice (FIG. 16).

Several studies have described the development and vaccine efficacy of attenuated mutant strains of *M. tuberculosis* (Jackson et al., 1999; Hondalus et al., 2000; Smith et al., 2001). The first study reported that a purine auxotroph of *M. tuberculosis* was unable to grow in macrophages and was attenuated for growth in both mice and guinea pigs (Jackson et al., 1999). A guinea pig vaccination experiment determined that a single immunization with the auxotroph allowed the animals to restrict the growth of virulent *M. tuberculosis* in the lungs as well as a single immunization with wild-type BCG, following aerosol challenge. However, the reduction in growth of the challenge organism in the spleen afforded by the auxotroph was not as extensive as that afforded by BCG. Another study reported that a leucine auxotroph of *M. tuberculois* Erdman cannot grow in macrophages and is avirulent to immunocompromised SCID mice (Hondalus et al., 2000). Immunocompetent mice vaccinated once with a *M. tuberculosis* leucine mutant did not significantly restrict the growth of the virulent challenge organism in the lungs or spleen as much as the control mice vaccinated with BCG (Id.). However, the mice immunized with the leucine auxotroph survived as long as the BCG immunized controls and exhibited a decreased histopathology relative to that seen in the non-immunized controls (Id.) A third study showed that M. tuberculosis proline and tryptophan auxotrophs were attenuated and a single immunization of mice with either of these

mutants afforded protection against an intravenous challenge with virulent *M. tuberculosis*, comparable to that for BCG, as indicated by the mean survival times (Smith et al., 2001). In those experiments, mice immunized with *pro* or *trp* mutants could restrict the growth of the challenge organisms to the same extent as mice immunized with BCG, although the magnitude of protection in either case (*M. tuberculosis* auxotrophs or BCG) was not as extensive as that seen in the other studies (Id.).

In the present study we have demonstrated that a single immunization of mice with the avirulent *M. tuberculosis* lysine auxotroph did not generate an immune response capable of significantly restricting the growth of virulent *M. tuberculosis* Erdman following an aerogenic challenge. However, administration of a second or a third dose of this vaccine increased protection substantially, as measured by the number of viable bacteria per organ, to a level similar to that achieved with single dose of BCG-Pasteur. This level of protection did not seem to be greatly increased by a third dose of vaccine, although the triply immunized mice survived as long as the control mice immunized with a single dose of BCG-Pasteur. Mice that were immunized twice were not followed to determine mean survival time, but comparing the growth curves of the challenge bacteria following the double and triple immunizations, it seems likely that the survival time for the doubly immunized mice would be much the same as that for the triple-immunized mice.

The previous studies using *M. tuberculosis* auxotrophs as vaccine strains showed substantial variations in their effectiveness. This variability is likely to be due to a number of factors, including the different *M. tuberculosis* background strains used to construct the mutants, different mouse strains used in the various protection studies, and the different challenge organisms and challenge routes used. There was also considerable variation in the protective efficacy of the different vaccines compared to that observed in controls using BCG immunization. These differences pose a number of questions concerning the best indicators of protection, especially in the long term. Should viable bacterial counts or survival be the primary indicator of protection or should both be given equal weight? The results of this study indicate that more than one immunization with a *M. tuberculosis* lysine auxotroph did generate a significant protective response as indicated by both criteria. We believe it is important that multiple immunization protocols be considered in the further development of attenuated *M. tuberculosis* strains as potential human vaccines.

This is the first study demonstrating that a multiple immunization protocol using an auxotroph of *M. tuberculosis* can protect against a highly virulent aerosol challenge compared

to that seen for BCG. Since BCG vaccines have shown variable efficacy when tested in humans, an auxotrophic *M. tuberculosis* vaccine might represent an attractive booster vaccine with which to augment childhood BCG immunization.

Example 6. Mutants of *Mycobacterium tuberculosis* having two attenuating mutations are safe and provide protection in mammals lacking $CD4^+$ lymphocytes.

The experiments described in this Example employ materials and methods described in the other Examples.

<u>Construction and characterization of *M. tuberculosis* Δ*RD1*Δ*panCD* (mc²6030).</u> A pantothenate auxotroph of *M. tuberculosis* Δ*RD1* was generated by specialized transduction and the strain designated mc²6030. No CFU were detected on 7H11 when $5x10^{10}$ CFU were plated (repeated twice), suggesting the reversion frequency to be below 10^{-11}.

SCID mice infected with $1x10^2$ CFU H37Rv succumbed to infection in 6 weeks, whereas the mice infected with $1x10^6$ mc²6030 survived significantly longer with more than 75% of mice surviving for more than 300 days (FIG. 17A). Bacteria isolated from mc²6030-infected mice before they died were all auxotrophs, confirming that there were no revertants under in vivo conditions. In order to assess the safety of mc²6030 in immunocompetent BALB/c mice, we infected mice intravenously with $1x10^6$ mc²6030 or $1x10^6$ of wild-type H37Rv. All mice infected with H37Rv succumbed to infection by 150 days, whereas mice infected with mc²6030 survived for more than 300 days (FIG. 17B). In an effort to understand the role of immune responses in controlling infection with the pantothenate mutants, we infected immunocompetent C57Bl/6 with $1x10^6$ CFU of mc²6001 (Δ*RD1*), mc²6004 (complementing strain), mc²6030 (Δ*RD1*Δ*panCD*) or wild-type H37Rv. Mice infected with H37Rv and mc²6004 showed progressive growth in all the three organs, whereas mice infected with mc²6030 showed a drop in growth during the first 3 weeks in the lungs and spleen (FIG. 18). Following 3 weeks of infection, the growth pattern of both mc²6001 and mc²6030 were identical in the spleen and lungs. Mice immunized subcutaneously with one or two doses of mc²6030 demonstrated protection against aerosol challenge with virulent *M. tuberculosis*, which was comparable to the protection afforded by BCG vaccination (Table 6). No pantothenate auxotrophs were recovered from spleen or lungs of mice at 1, 2 or 3 months following subcutaneous immunization.

Table 6. Bacterial burden of virulent *M. tuberculosis* in uninoculated mice and mice inoculated with BCG or one or two doses of Δ*RD1*Δ*panCD*.

Experimental Group	Lung CFUs ($\log_{10}$)	Spleen CFUs (log10)
Naive	5.99 ± 0.09	4.94 ± 0.06
Δ*RD1*Δ*panCD* (1 dose) sc	5.22 ± 0.10*	4.04 ± 0.15*
Δ*RD1*Δ*panCD* (2 doses) sc	4.86 ± 0.14**	3.58 ± 0.11**
BCG (1 dose) sc	4.79 ± 0.19**	3.73 ± 0.27**

*p<0.01 relative to controls; **p<0.001 relative to controls

<u>Construction and characterization of *M. tuberculosis* Δ*lysA*Δ*panCD* (mc²6020).</u> A pantothenate auxotroph of *M. tuberculosis* Δ*lysA* was generated by specialized transduction and the strain designated mc²6020. No CFU were detected on 7H11 when $5x10^{10}$ CFU were plated, suggesting the reversion frequency to be below 10^{-11}. This double mutant is auxotrophic for both lysine and pantothenate. SCID mice infected with $1x10^2$ CFU H37Rv succumbed to infection in 6 weeks, whereas the mice infected with $1x10^6$ mc²6020 survived for more than 400 days with no mortality. In order to assess the safety and growth kinetics of mc²6020 in immunocompetent BALB/c mice, we infected mice intravenously with $1x10^6$ mc²6020 or $1x10^6$ of wild-type H37Rv. All mice infected with H37Rv succumbed to infection by 150 days, whereas mice infected with mc²6020 survived for more than 400 days. After 3 weeks following intravenous infection, no colonies of mc²6020 could be recovered from spleen, liver or lungs of infected mice. Interestingly, mice immunized subcutaneously with one or two doses of mc²6020 demonstrated protection against aerosol challenge with virulent *M. tuberculosis*, which was comparable to the protection afforded by BCG vaccination (Table 7). No pantothenate and lysine requiring auxotrophs were recovered from spleen or lungs of mice at 1, 2 or 3 months following subcutaneous immunization. Other studies established that both mc²6020 and mc²6030 protects the a level of protection of mice against TB equivalent to the protection afforded by BCG (FIG. 19).

Table 7. Bacterial burden of virulent *M. tuberculosis* in uninoculated mice and mice inoculated with BCG or one or two doses of mc[2]6020 (Δ*lysA*Δ*panCD*) sc or one dose of mc[2]6020 iv.

Experimental group	Lung CFUs ($\log_{10}$)	Spleen CFUs ($\log_{10}$)
naive	6.03 ± 0.05[a]	4.84 ± 0.27
BCG (1 dose) sc	4.76 ± 0.19***	3.95 ± 0.18*
mc[2]6020 (1 dose) sc	5.05 ± 0.06***	4.02 ± 0.11*
mc[2]6020 (2 doses) sc	5.09 ± 0.05***	4.06 ± 0.27
mc[2]6020 (1 dose) iv	5.06 ± 0.11***	4.00 ± 0.15*

[a]Mean ± SEM $p<0.001$ = ***; $p<0.05$ = *

These data clearly demonstrate the safety and immunogenicity of these two double mutants of *M. tuberculosis* in mice.

The double deletion mutant mc[2]6030 (Δ*RD1*Δ*panCD*) immunizes and protects CD4$^{-/-}$ mice from aerosolized *M. tuberculosis* challenge. We tested the hypothesis that the attenuated double deletion mutants could protect CD4-deficient mice, a model of HIV-infected humans, from aerosolized *M. tuberculosis* challenge. The results of these tests are summarized in FIG. 20. While 100% of the non-immunized CD4$^{-/-}$ were dead by 38 days, 100% of the mice immunized with either BCG or mc[2]6030 are alive at 120 days post challenge. After 120 days, none of the immunized mice had any outward sign of disease. The double deletion mutants were also safer than BCG in SCID mice, where all of the SCID mice died before 100 days when inoculated with BCG, 100% and 25% of the mice survived inoculation with mc[2]6020 and mc[2]6030, respectively (FIG. 21). This indicates that immunity against *M. tuberculosis* can be elicited in a CD4-independent manner. These results also support the notion that effective antibody or CD8-mediated vaccines to malaria and HIV could be developed in the context of these attenuated *M. tuberculosis* strains.

Example 7. *Mycobacterium tuberculosis* RD1 panCD Is safe and protects CD4-deficient mice against tuberculosis in the absence of CD8 cells

Example Summary

Tuberculosis (TB) remains a leading cause of death due to an infectious agent and is particularly devastating among HIV-infected individuals. The risk of disseminated BCG

disease precludes the use of BCG vaccine in adults with known HIV infection or children with symptomatic AIDS. There is an urgent need for a safe and effective vaccine against tuberculosis for immunocompetent, as well as immunodeficient individuals. Here, we report that a mutant of *Mycobacterium tuberculosis* with two independent deletions, in the *RD1* region and *panCD* genes, is severely attenuated in immunocompromised mice lacking T and B cells or mice lacking interferon-gamma and significantly safer than the BCG vaccine. A single subcutaneous immunization with the Δ*RD1* Δ*panCD* mutant induces significant protective immune responses that prolong the survival of immunocompetent mice following a challenge with virulent M. tuberculosis. As a model that reflects the loss of CD4-cells associated with HIV infection, we tested whether *M. tuberculosis* Δ*RD1* Δ*panCD* could immunize and protect CD4-deficient mice against aerosol challenge with virulent *M. tuberculosis*. Surprisingly, immunization with this mutant affords significantly enhanced post-challenge survival to CD4-deficient mice than BCG vaccine. Furthermore, treatment of Δ*RD1* Δ*panCD* vaccinated $CD4^{-/-}$ mice with anti-CD8 antibody did not eliminate the protection, suggesting the role of a novel class of $CD4^{-}CD8^{-}$ cells in mediating this protection. Our results highlight the feasibility of generating multiple deletion mutants of *M. tuberculosis* that are non-revertible, highly safe and yet retain the ability to induce strong protective immunity against TB in both immunocompetent and CD4-deficient mice.

Introduction

The global problem of tuberculosis (TB) is worsening, primarily as a result of the growing HIV pandemic (Corbett et al., 2003). TB is a leading cause of death due an infectious agent, claiming more than 2 million lives each year, with approximately 12% attributable to HIV. The global TB problem is further worsened by the emergence of multi-drug resistant strains of *Mycobacterium tuberculosis* (Pablos-Mendez et al., 1998). Clearly, novel interventions in the form of an effective vaccine are urgently needed to reduce the disease burden of TB, particularly for HIV-infected individuals.

Vaccination with bacille Calmette-Guérin (BCG), a live attenuated strain of *Mycobacterium bovis*, induces protective immunity in children against severe and fatal forms of TB (Bloom and Fine, 1994; Rodrigues et al., 1993). However, the protection afforded by BCG vaccine against the most prevalent pulmonary form of TB in adults is highly variable (0 to 80%) (Tuberculosis Prevention Trail, 1980; Fine, 1995). Although BCG has been administered to >3 billion people and has an overall excellent safety record (Lotte et al., 1988), there have been several cases of disseminated BCG disease in individuals with mutations of

their IL-12, or IL-12R genes following vaccination or infection with BCG (Altare et al., 1998; Casanova et al., 1995; de Jong et al., 1998). Likewise, there have been numerous cases of disseminated BCG have been detected in vaccinated children who subsequently developed AIDS (Talbot et al., 1997; von Reyn et al., 1987; Weltman and Rose, 1993; Braun and Cauthen, 1992). Therefore, a safer and more effective vaccine than the currently used BCG vaccine is urgently needed to control TB in HIV-infected individuals.

The genetic basis for the primary attenuation of the widely used *M. bovis* derived BCG vaccine has been attributed to the loss of approximately 10 genes, named the region of difference, RD1 region (Lewis et al., 2003; Pym et al., 2002; Hsu et al., 2003). Comparative genomic studies have revealed at least 129 open reading frames to be missing from BCG strains in comparison to wild-type *M. tuberculosis* (Mahairas et al., 1996; Behr et al., 1999; Gordon et al., 1999). These missing regions may encode potential antigenic determinants that could increase the immunogenicity of a vaccine, if it were derived from an attenuated strain of *M. tuberculosis*. Several live, attenuated *M. tuberculosis* vaccine candidates have been constructed by deleting genes required for growth in mice (Hondalus et al., 2000; Jackson et al., 1999; Smith et al., 2001) and have shown to confer some degree of protection against challenge infection with virulent *M. tuberculosis*.

We had previously reported the significant safety and immunogenicity of a Δ*panCD* mutant of *M. tuberculosis* in mice (Sambandamurthy et al., 2002). In an attempt to further enhance the safety of a *M. tuberculosis* derived vaccine, we deleted the *panCD* genes from the Δ*RD1* mutant of *M. tuberculosis* that contains at least three attenuating mutations (Hsu et al., 2003). The resulting Δ*RD1* Δ*panCD* mutant has the safety features of two independent nonrevertible genomic deletions, which confer significant attenuation even in immunodeficient mice that lack T and B cells or mice that lack γ-interferon. In addition, the Δ*RD1* Δ*panCD* mutant undergoes limited replication *in vivo*; and thus confers significant long-term protection and survival in mice following a single dose vaccination. We also demonstrate that this attenuated TB vaccine is significantly better than BCG in prolonging the survival of CD4-deficient mice following an aerosol challenge with virulent *M. tuberculosis* and the protective immunity induced in these immunocompromised mice is largely mediated by a novel class of $CD4^-CD8^-$ cells.

Materials and Methods

Media and Strains. *M. tuberculosis* H37Rv, *M. tuberculosis* Erdman and *M. bovis* BCG Pasteur were obtained from the Trudeau Culture Collection (Saranac Lake, NY) and

cultured as described earlier (Sambandamurthy et al., 2002). When required, pantothenate (24 μg/ml) or hygromycin (50 μg/ml) was added. Stock strains were grown in Middlebrook 7H9 broth in roller bottles and harvested in mid-logarithmic growth phase, before being stored in 1 ml vials at -70° C.

Construction of M. tuberculosis Δ*RD1* Δ*panCD* (mc26030) deletion mutant. Specialized transduction was employed to disrupt the chromosomal copy of the *panCD* genes from the unmarked *M. tuberculosis* Δ*RD1* mutant (Hsu et al., 2003). The *panCD* and *RD1* deletions were confirmed using PCR and Southern blotting as described earlier (Hsu et al., 2003; Sambandamurthy et al., 2002).

Animal infections. C57BL/6, BALB/c, BALB/c SCID, C57BL/6 GKO (6-8 weeks old) were purchased from Jackson Laboratories and were infected intravenously through the lateral tail vein. For time-to-death and mouse organ CFU assays, BALB/c SCID mice were infected intravenously with 10^2 CFUs of *M. tuberculosis* H37Rv or 10^5 CFUs of mc^{2}6030. For GKO survival experiments, mice were infected intravenously with 10^5 CFUs of *M. tuberculosis* H37Rv, BCG-P or the mc^{2}6030 mutant. For mouse organ CFU assays, C57BL/6 mice were infected with 10^6 CFUs of *M. tuberculosis* H37Rv or the mc^{2}6030 mutant. At appropriate time points, groups of 4 or 5 mice were sacrificed and the bacterial burden estimated as described earlier (Sambandamurthy et al., 2002). The survival data and the CFU results were statistically evaluated using either one-way analysis of variance or unpaired t-test analyses provided by the GraphPad InStat program. Pathologic examination was performed on tissues fixed in 10% buffered formalin. All animals were maintained in accordance with protocols approved by the Albert Einstein College of Medicine and the Center for Biologics Evaluation and Research Institutional Animal Care and Use Committee.

Vaccination Studies. Groups of C57BL/6 mice (5 mice per group) were vaccinated subcutaneously, either once or twice (6 weeks apart), with 10^6 CFUs of mc^{2}6030. Control mice were vaccinated subcutaneously with 10^6 CFUs of *M. bovis* BCG-Pasteur. At either 3 or 8 months after the initial immunization with mc^{2}6030 or BCG, the mice were aerogenically challenged with approximately 100 - 200 CFUs of M. tuberculosis Erdman strain. At 28 days following aerosol challenge, the challenged mice were sacrificed and the bacterial numbers in the lung and spleen were enumerated as described earlier (Sambandamurthy et al., 2002).

For immunization studies in CD4$^{-/-}$ mice (Jackson Laboratories), mice were vaccinated subcutaneously with a single dose of 10^6 CFUs of mc^{2}6030 or BCG-P and challenged three months later through the aerosol route. For the survival studies, 5 to 6 mice per group were

maintained until they became moribund and had to be euthanized. Five mice were sacrificed after 24 hours to confirm the size of the challenge dose following the aerosol challenge.

For the CD8 depletion studies, $CD4^{-/-}$ mice were immunized with a single subcutaneous dose of $mc^2$6030 and then treated intraperitoneally with anti-CD8 monoclonal antibody (clone 243, Harlan Bioproducts, Indianapolis IN) at two days before, the day of the tuberculous challenge, and then twice per week (0.2 mg/ml per dose) until the mice were sacrificed. The effectiveness of the anti-CD8 antibody treatment was confirmed by flow cytometry after staining peripheral blood and lung lymphocytes with Cy-Chrome-conjugated rat anti-mouse CD8a (ly-2) monoclonal antibody (Pharmingen, San Diego, CA).

To assess the role of IFN-γ in mediating protection, GKO mice (n = 10) were vaccinated with 10^6 CFU of $mc^2$6030 or BCG-P and challenged three months later through the aerosol route and followed for survival.

Results

$mc^2$6030 is highly attenuated in immunodeficient mice. In an attempt to develop a safe and effective vaccine strain, the *panCD* genes (Sambandamurthy et al., 2002) were deleted from an unmarked Δ*RD1* mutant of *M. tuberculosis* using specialized transduction (Bardarov et al., 2002). The deletions in the *RD1* region and *panCD* genes were confirmed using PCR and Southern blotting (data not shown). Strain $mc^2$6030 is auxotrophic for pantothenate; no revertants were recovered when 10^{11} CFU of $mc^2$6030 strain were plated on minimal media, demonstrating the mutations to be highly stable and non-revertible.

An important pre-requisite for any live attenuated vaccine is their safe use even in immunodeficient hosts. To assess the attenuation of this mutant, severe combined immunodeficient (SCID) mice, a highly stringent model for safety, were infected intravenously with H37Rv or $mc^2$6030. Mice infected with 10^2 CFU of *M. tuberculosis* H37Rv strain died within 4 weeks post-infection. Interestingly, 60% of SCID mice infected with 10^5 CFU of $mc^2$6030 survived for over 350 days (FIG. 22a). Mice infected with H37Rv showed a rapid increase in bacterial numbers in the lungs, spleen and liver by 3 weeks. In contrast, the bacterial numbers in the spleen of $mc^2$6030-infected mice remained relatively constant throughout the course of infection (FIG. 22b). Bacterial numbers in the lungs of $mc^2$6030-infected SCID mice showed a decrease in the first 3 weeks of infection, but gradually increased to reach 10^8 CFUs by 350 days (FIG. 22c). The bacterial titers were constant in the liver throughout the course of infection except for a sharp decline at 3 weeks (data not shown).

Overwhelming evidence from humans and animal models has implicated IFN-γ to be a key cytokine in the control of *M. tuberculosis* infection (Flynn and Chan, 2001). IFN-γ knockout (GKO) mice are extremely sensitive to tuberculous infection (Cooper et al., 1993; Flynn et al., 1993) and individuals defective in genes for IFN-γ receptors have increased susceptibility to disseminated mycobacterial disease (Ottenhoff et al., 1998). As an additional assessment of the safety of this attenuated mutant, GKO mice were infected intravenously with 10^5 CFU of *M. tuberculosis* H37Rv, BCG Pasteur (BCG-P) or mc^{2}6030. All of the GKO mice infected with H37Rv (mean survival time, MST, 21 days) or BCG-P (MST, 93 days) succumbed to the tuberculous infection within 4 months, whereas >60% of mice infected with mc^{2}6030 were alive at 335 days (FIG. 22d). The extreme susceptibility of immunocompromised mice to *M. tuberculosis* and BCG infections is consistent with data from previous studies using SCID (Sambandamurthy et al., 2002; Weber et al., 2000) and GKO mice (Dalton et al., 1993). Overall, our data indicate that mc^{2}6030 is significantly more attenuated and less virulent in SCID and GKO mice than the widely used BCG vaccine strain.

<u>mc^{2}6030 undergoes limited replication in mice.</u> To evaluate the effect of the multiple mutations in strain mc^{2}6030 on bacterial growth in vivo, immunocompetent BALB/c or C57BL/6 mice were infected intravenously. All BALB/c mice infected with 10^5 CFU of H37Rv succumbed to the resulting infection by 168 days (MST = 134 days). In contrast, all mice infected with 10^5 CFU of mc^{2}6030 survived for over 400 days (data not shown). Similarly, C57BL/6 mice infected with 10^6 CFU of H37Rv succumbed to infection by 260 days (MST = 196 days). All mice infected with 10^6 CFUs of mc^{2}6030 survived over 400 days post-infection (FIG. 22e). Bacteriological culture results generally showed a decline in the numbers of H37Rv and mc26030 organisms in the spleen (FIG. 22f) and liver (data not shown) of C57BL/6 mice after infection. Interestingly, the pulmonary bacterial CFU numbers for both the virulent H37Rv strain and the attenuated mutant reached a constant level at 3 months post-infection (FIG. 22g). Importantly, the H37Rv CFU values were at least 100-fold higher than the mc^{2}6030 CFUs in all organs tested at 200 days after the infection.

Histopathological examination of organs from infected mice confirmed the marked attenuation of the deletion mutant. At 3 weeks post-infection, an intravenous injection of mc^{2}6030 (FIG. 22h) had caused only rare, mild perivascular, lymphocytic infiltrates. The spleens were slightly enlarged with mononuclear cell infiltration in the red pulp. Also, multifocal, mild infiltrations of macrophages and neutrophils were seen in the liver and no acid-fast bacilli were detected. This contrasted with the severe pneumonia (FIG. 22i),

markedly enlarged spleens, severe diffuse granulomatous hepatitis, and the overwhelming bacterial burden seen in mice infected with the H37Rv strain.

mc^{2}6030 induces short and long-term protection in immunocompetent mice. Having assessed the safety and growth kinetics of mc^{2}6030 in both immunodeficient and immunocompetent mice, we evaluated the protective immune responses induced by this attenuated strain. As a test of its vaccine potential, C57BL/6 mice were immunized subcutaneously with 10^6 CFUs of mc^{2}6030 and then were challenged 3 months later with a low dose of virulent *M. tuberculosis* Erdman by the aerosol route. Following subcutaneous immunization, the immunizing mc^{2}6030 mutant bacteria could not be cultured from the spleens or lungs of mice at 8 and 12 weeks postvaccination. At 28 days post-aerosol challenge, mice that were immunized 3 months earlier with a single dose of mc^{2}6030 showed a significant reduction in the lung ($P < 0.01$) and spleen ($P < 0.01$) bacterial CFU values as compared to naïve mice. Consistent with published results, mice vaccinated with 10^6 CFUs of BCG showed similar CFU reductions in the lungs ($P < 0.001$) and spleen ($P < 0.001$) as compared to the naïve controls (FIG. 23a,b).

In order to assess the duration and persistence of the memory immune response, vaccinated and control mice were challenged through the aerosol route 8 months after a single dose vaccination. In the naïve controls at four weeks post-challenge, the bacterial numbers increased dramatically in the lung and substantial dissemination and growth in the spleen were also observed. Strikingly, mice vaccinated with a single dose of mc^{2}6030 or BCG-P displayed statistically significant reduction in bacterial numbers in the lungs ($P < 0.001$) and spleen ($P < 0.001$) relative to naïve controls (FIG. 23c,d). Our data clearly demonstrate that a single dose of mc^{2}6030 induces a potent and long-lasting protective immune response that is effective in controlling a virulent *M. tuberculosis* challenge in the lungs and spleen of mice even after 8 months following the primary immunization.

Another relevant measure of vaccine effectiveness is the relative survival periods for immunized mice following a challenge with virulent organisms (33). To evaluate whether vaccination with mc^{2}6030 increased mean survival times, C57BL/6 mice that had been immunized subcutaneously with a single dose of mc^{2}6030 (or BCG-P) were challenged 3 months later with virulent *M. tuberculosis* Erdman by the aerosol route and followed for survival. All of the unvaccinated mice succumbed to the tuberculous infection (MST = 95 ±22 days) following the aerosol challenge. In contrast, the survival periods of mice vaccinated with

either $mc^2 6030$ (MST, 267±14 days) or BCG-P (MST, 268 ±16 days) were significantly extended ($P < 0.001$) relative to naïve mice (FIG. 23e).

mc26030 protects CD4-deficient mice significantly better than BCG against anaerosolized TB challenge. Tuberculosis remains the largest attributable cause of death in HIV-infected individuals (Whalen et al., 2000). HIV infection leads to a loss of $CD4^+$ T cells and previous studies have demonstrated that mice deficient in $CD4^+$ T cells are highly susceptible to *M. bovis* BCG (Ladel et al., 1995) and *M. tuberculosis* infection (Mogues et al., 2001; Caruso et al., 1999). Since BCG vaccination is contraindicated in HIV-infected individuals, we wanted to test if the more attenuated strain, $mc^2 6030$, could protect CD4-deficient mice from experimental tuberculosis. CD4-deficient mice were vaccinated subcutaneously with a single dose of 10^6 CFUs of either $mc^2 6030$ or BCG-P and then aerogenically challenged with 100-200 CFUs of *M. tuberculosis* Erdman three months later. At 28 days post-aerosol challenge, the bacterial burden in the lungs ($P < 0.001$) and spleen ($P < 0.001$) of the $mc^2 6030$ and BCG-P vaccinated mice was decreased by greater than 99% (>2 $\log_{10}$ CFU) relative to naïve controls (FIG. 24a,b).

To further evaluate the long-lived protection induced by $mc^2 6030$, $CD4^{-/-}$ mice were vaccinated, challenged 3 months later with virulent *M. tuberculosis* Erdman through the aerosol route, and followed for survival. All of the naïve mice died within 29 days (MST = 27 ±2 days) of the low dose tuberculous aerogenic challenge (FIG. 24c). Strikingly, the mean survival time for the $CD4^{-/-}$ mice vaccinated with a single dose of $mc^2 6030$ was 214 ±18 days, a nearly eight-fold extension of the survival period compared to naïves. In contrast, the BCG vaccinated mice survived 158 ±23 days. The 56-day extension of the MST for the $mc^2 6030$-immunized mice relative to BCG vaccinated animals represented a significantly improved outcome ($P < 0.05$) for $CD4^{-/-}$ mice immunized with the *M. tuberculosis* mutant strain.

Histologically, significant differences were observed between the vaccinated groups and naïve $CD4^{-/-}$ controls. The unvaccinated mice developed severe lung lesions with multiple large inflammatory nodules; some coalescing and spreading to areas of extensive diffuse pneumonia. The inflammatory response consisted of macrophages, numerous neutrophils, accompanied by low numbers of lymphocytes. There were large numbers of acid-fast organisms in the lesions (FIG. 25a,d). In contrast, mice vaccinated with $mc^2 6030$ (FIG. 25b,e) or BCG (FIG. 25c,f) showed reduced severity of the lung lesions. These mice showed scattered distinct lesions, adjacent to airways and localized, which remained smaller in

diameter than in unvaccinated mice. These multifocal areas were composed of macrophages and numerous lymphocytes. There was a marked reduction in the numbers of acid-fast organisms compared to naïve controls.

The ability of mc^26030-vaccinated $CD4^{-/-}$ mice to control the tuberculous challenge suggests the potential role of $CD8^+$ T cells in mediating this protection. In order to directly demonstrate the role of $CD8^+$ T cells in this protective response, $CD4^{-/-}$ mice that were vaccinated with mc^26030 were treated with anti-CD8 monoclonal antibody and subsequently challenged with virulent *M. tuberculosis* through the aerosol route. Flow cytometric analysis showed that the anti-CD8 antibody treatment had depleted >99% of $CD8^+$ T cells from the lungs and peripheral blood. Surprisingly, repeated injections of the anti-CD8 antibody did not reduce the vaccine-mediated protective immune response. At 28 days post-aerosol challenge, the bacterial burdens in the lungs and spleens of the anti-CD8 antibody-treated immunized $CD4^{-/-}$ mice and nontreated vaccinated mice were similar; significant reductions in the pulmonary and splenic bacterial CFUs, relative to nonimmunized controls (>2 $\log_{10}$ in the lungs and >1 $\log_{10}$ in the spleen) were detected for each vaccine group (FIG. 24d,e).

To examine the role of IFN-γ in mediating the anti-tuberculous immunity evoked by the mutant *M. tuberculosis* strain, GKO mice were vaccinated with mc^26030 or BCG-P and challenged with *M. tuberculosis*. Immunization of GKO mice with mc^26030 or BCG-P did not significantly increase the survival period in comparison to unvaccinated controls with all mice in each of the three groups succumbing to the resulting infection within 30 days. Interestingly, 4 out of 10 BCG-vaccinated GKO mice died of disseminated BCG infection even before the aerosol challenge (FIG. 24f).

Discussion

Vaccination remains the most cost-effective and proven strategy to protect mankind against infectious agents (Bloom, 1989). The eradication of smallpox and the near elimination of poliomyelitis demonstrate the potential of one class of vaccines, the live attenuated vaccines. However, a major obstacle to the development of these vaccines is the difficulty in achieving a satisfactory level of attenuation without severely compromising the immunogenicity of the vaccine strain. Recent advances in the molecular biology of mycobacteria have been used to construct several attenuated mutants of *M. tuberculosis* as candidates for a vaccine (Hondalus et al., 2000; Jackson et al., 1999; Smith et al., 2001). An *M. tuberculosis*-derived vaccine may be more efficacious and induce a qualitatively better protective immune response than an *M. bovis*-derived BCG vaccine because the

immunogenicity of BCG seem to have declined following extensive passage over the years (Behr and Small, 1997) and *M. tuberculosis* mutants should express an antigenic profile that is nearly identical to virulent TB.

The deletion of the *RD1* region (the primary attenuating deletion in all BCG vaccine strains) from *M. tuberculosis* leads to attenuation of the resulting mutant strain (Lewis et al., 2003; Hsu et al., 2003). However, that strain is relatively more virulent than the currently used BCG vaccine in immunodeficient SCID mice. In contrast, a multiple deletion mutant of *M. tuberculosis* (mc^{2}6030) harboring deletions in the *RD1* region and the *panCD* genes is severely attenuated in SCID mice. This mutant is considerably less virulent than the single Δ*RD1* or Δ*panCD* *M. tuberculosis* mutants or BCG in this mouse model. Interestingly, our data also show that the mc^{2}6030 mutant is markedly more attenuated than the widely used BCG vaccine in mice lacking IFN-γ.

While live strains must be attenuated, they should retain their capacity to undergo limited *in vivo* replication (McKenney et al., 1999; Kanai and Yanagisawa, 1955; Brant et al., 2002). The failure of earlier auxotrophs of *M. tuberculosis* to induce a potent protective immune response could be due to insufficient *in vivo* replication. The capacity of mc^{2}6030 mutant to undergo limited replication without causing detrimental pathology in the tissues of infected wild-type mice likely contributes to its ability to generate long-term immunity in mice. The hallmark of immunological memory and vaccination is the ability to mount an accelerated response to reinfection, which is achieved by the generation of long-lived memory T cells. The induction and long-term maintenance of cellular immune responses is the primary goal of a vaccine for an intracellular pathogen such as *Mycobacterium tuberculosis* (Seder and Hill, 2000). For the mc^{2}6030 strain, the protective response seen at 8 months following a single immunization reflects the generation of long-term memory response. An additional proof of this induction of immunological memory was demonstrated in the survival studies; mice vaccinated with mc^{2}6030 survived significantly longer than control mice following a tuberculous challenge.

HIV-infection is a major risk factor for both primary and reactivational disease with *M. tuberculosis* in humans. The annual risk of developing TB in individuals coinfected with TB and HIV is 10%, while immunocompetent individuals have only a 10% lifetime risk of developing disease following infection. Therefore, HIV-1 infected individuals remain a critical cohort of patients suitable for vaccination. However, candidate vaccines may not be sufficiently immunogenic to stimulate protective immunity in potentially immunodeficient

HIV-1 infected patients with impaired or defective CD4$^+$ T cell responses. To test this principle, we explored whether immunedeficient mice lacking CD4$^+$ T cells could be protected against TB by vaccination with a safe mutant of *M. tuberculosis*. Interestingly, our study clearly demonstrates the ability of a single dose vaccination with mc^{2}6030 to confer significant protection in CD4$^{-/-}$ mice following an aerosol challenge with virulent *M. tuberculosis*. Reduced organ bacterial burdens, prolonged survival and a milder pulmonary histopathology were seen in vaccinated mice relative to naïve mice lacking CD4$^+$ T cells. Importantly, the mc^{2}6030 vaccinated CD4$^{-/-}$ mice survived significantly longer (56 days) post-challenge than BCG-vaccinated animals. The immunogenicty of this mutant and its reduced virulence likely contribute to the improved outcome in these immunocompromised mice. Overall, these data strongly suggest that further testing of this attenuated *M. tuberculosis* strain in other immunocompromised models is warranted with a goal of developing an effective TB vaccine in HIV-1 infected individuals.

The biological basis for the enhanced effectiveness of the mc^{2}6030 strain in mice lacking CD4$^+$ T cells has not been completely elucidated. Clearly, the specific immune mechanisms associated with protection after vaccination and challenge in CD4-deficient mice are of substantial interest because several studies have suggested that CD4$^+$ T cells are absolutely required to generate anti-tuberculosis protective immunity (Flynn and Chan, 2001). Since CD8$^+$ T cells have been shown to play an important role in the control of acute tuberculosis (Behar et al., 1999; Sousa et al., 2000; Serbina and Flynn, 2001) and persistent infection (van Pinxteren et al., 2000b), we hypothesized that CD8 cells mediated the protective immunity generated by vaccination of CD4$^{-/-}$ mice with the mc^{2}6030 strain. Although several studies have demonstrated that CD4$^+$ T cell help is important in the activation and differentiation of naïve CD8$^+$ T cells into cytotoxic effector cells (Cardin et al., 1996; van Herrath et al., 1996; Wild et al., 1999), recent evidence suggests that vaccine immunity can be generated in the absence of CD4$^+$ T cells (Buller et al., 1987; Stevenson et al., 1998; Wuthrich et al., 2003) and CD8$^+$ T cells can provide self-help when they are present at a sufficiently high precursor frequency (Wang et al., 2001; Mintern et al., 2002). Consistent with this premise, potent CD8 mediated anti-mycobacterial protective immunity was generated following immunization with a DNA vaccine cocktail in CD4-deficient mice (Steven Derrick and Sheldon Morris, Infection and Immunity, in press). Surprisingly, treatment of vaccinated mice with an anti-CD8 antibody did not reduce the protective immune response in CD4$^{-/-}$ mice induced by immunization with the *M. tuberculosis* mutant. This result suggests that CD4$^-$CD8$^-$

cells may contribute to the anti-tuberculous protection. These anti-CD8 antibody data are consistent with the previously described role of double-negative T cells in protecting against infections with intracellular pathogens. In a *Listeria* model, Dunn and North showed the resolution of primary listeriosis and acquired resistance to secondary infection to be predominantly mediated by double-negative T cells (Dunn and North, 1991). More recently, Cowley and Elkins reported that secondary immunity to the *Francisella tularensis* live vaccine strain was partially mediated by a unique $CD4^{-}CD8^{-}$ T cell population (Cowley and Elkins, 2003). Further studies are currently in progress to directly determine whether double-negative T cells are mediating the Δ*RD1* Δ*panCD* vaccine strain-induced protective immunity in CD4-deficient mice and whether $CD4^{-}CD8^{-}$ T cells are generally involved in the protective responses elicited by live attenuated strains against intracellular pathogens.

Overall, our results demonstrate that it is possible to generate two independent unlinked deletions, each containing multiple attenuating mutations into *M. tuberculosis* to generate a safe mutant that can remain immunogenic and provide protective immunity against airborne infection with virulent *M. tuberculosis* in mice. The protection and safety data from immunocompromised and immunocompetent mice and the lack of reversion make this multiple deletion mutant a viable vaccine candidate for humans. The numerous advantages of BCG, i.e., affordability, safety, ability to be used in newborns, and its use as a recombinant vaccine delivery vector should be also applicable to the highly attenuated $mc^{2}6030$ candidate. Since the $mc^{2}6030$ strain is more attenuated than BCG and yet is more protective in immunocompromised mice, the evaluation of the $mc^{2}6030$ mutant as a vaccine in other animal models of tuberculosis, including nonhuman primate models, is clearly warranted.

In view of the above, it will be seen that the several advantages of the invention are achieved and other advantages attained.

As various changes could be made in the above methods and compositions without departing from the scope of the invention, it is intended that all matter contained in the above description and shown in the accompanying drawings shall be interpreted as illustrative and not in a limiting sense.

All references cited in this specification are hereby incorporated by reference. The discussion of the references herein is intended merely to summarize the assertions made by the authors and no admission is made that any reference constitutes prior art. Applicants reserve the right to challenge the accuracy and pertinence of the cited references.

SEQ ID NO:s

SEQ ID NO:1 - An RD1 region of *Mycobacterium tuberculosis* H37Rv.

Bases 4350263-4359716 of the genome of *M. tuberculosis* H37Rv, as provided in GenBank Accession No. NC000962.

4350263 gatcgtgg gtgccgccgg ggggatgccg ccgatggcac
4350301 cgctggcccc gttattgccg gcggcggcag atatcgggtt gcacatcatt gtcacctgtc
4350361 agatgagcca ggcttacaag gcaaccatgg acaagttcgt cggcgccgca ttcgggtcgg
4350421 gcgctccgac aatgttcctt tcgggcgaga agcaggaatt cccatccagt gagttcaagg
4350481 tcaagcggcg cccccctggc caggcatttc tcgtctcgcc agacggcaaa gaggtcatcc
4350541 aggcccccta catcgagcct ccagaagaag tgttcgcagc acccccaagc gccggttaag
4350601 attatttcat tgccggtgta gcaggacccg agctcagccc ggtaatcgag ttcgggcaat
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4350721 agggagggaa gaagtaggca aatggaaaaa atgtcacatg atccgatcgc tgccgacatt
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4358161 ttcggcaatt ccatcccgtc gggtatgtag gccagcccat agctgttggc cacgacgatg
4358221 gaaccgtcgg tggtcaccgc ggtgatccag aagaacccgt agtcgcccgc gttgttgtcg
4358281 gacgcgttga gcgccgccgc gatgcgtcgc gccaaccgca gcgcatcacc gcggccacgc
4358341 tggcgggcgc tggcagctgc agtggcggcg tcgcgtgccg cccgagccgc cgacaccggg
4358401 atcatcgaca ccggcgtacc gtcatctgca gactcgctgc gatcgggttt gtcgatgtga
4358461 tcggtcgacg gcgggcgggc aggaggtgcc gtccgcgccg aggccgcccg cgtgctcggt

4358521 gccgccgcct tgtccgaggt agccaccggc gcccgcccag tggcagcatg cgaccccgcg
4358581 cccgaggccg cggccgtacc cacgctcgaa cgcgcgcccg ctcccacggc ggtaccgctc
4358641 ggcgcggcgg ccgccgcccg tgcgcccggg acaccggacg ccgcagccgg cgtcaccgac
4358701 gcggcggatt cgtccgcatg ggcaggcccc gactgcgtcc ccccgcccgc atgctggccc
4358761 ggcacaccag gttgctccgc caacgccgcg ggtttgacgt gcggcgccgg ctcgcccccct
4358821 ggggtgcccg gtgttgctgg accagacgga ccgggagtgg ccggtgtaac cggctggggc
4358881 ccaggcgatg gcgccggtgc cggagccggc tgcgggtgtg gagcgggagc tggggtaacg
4358941 ggcgtggccg gggttgccgg tgtggccggg gcgaccgggg gggtgaccgg cgtgatcggg
4359001 gttggctcgc ctggtgtgcc cggtttgacc ggggtcaccg gggtgaccgg cttgccccggg
4359061 gtcaccggcg tgacgggagt gccgggcgtt ggtgtgatcg gagttaccgg cgctcccggg
4359121 atgggtgtga ttggggttcc cggggtgatc ggggttcccg gggtgatcgg ggttcccggt
4359181 gtgcccggtg tgcccgggga tggcacgacc agggtaggca cgtctggggg tggcggcgac
4359241 ttctgctgaa gcaaatcctc gagtgcgttc ttcggaggtt tccaattctt ggattccagc
4359301 acccgctcag cggtctcggc gaccagactg acattggccc catgcgtcgc cgtgaccaat
4359361 gaattgatgg cggtatggcg ctcatcagca tccaggctag ggtcattctc caggatatcg
4359421 atctcccgtt gagcgccatc cacattattg ccgatatcgg atttagcttg ctcaatcaac
4359481 ccggcaatat gcctgtgcca ggtaatcacc gtggcgagat aatcctgcag cgtcatcaat
4359541 tgattgatgt ttgcacccag ggcgccgttg gcagcattgg cggcgccgcc ggaccatagg
4359601 ccgccttcga agacgtggcc tttctgctgg cggcaggtgt ccaatacatc ggtgaccctt
4359661 tgcaaaacct ggctatattc ctgggcccgg tcatagaaag tgtcttcatc ggcttc

SEQ ID NO:2 - A *panCD* region of *Mycobacterium tuberculosis* H37Rv,deleted in the Δ*panCD* strain of Example 2.

GGTCTAGCAGCTCGCCCGCGTTTTCGGGCACAAATGCCGGATCGTGGCCCATGTCG
ATCGGTTTGTTGTAAGCGTCGACAAACACGATCCGCGGCTGGTATGTGCGGGCCCG
GGCGTCGTCCATCGTCGCGTACGCAATCAGAATCACCAGATCCCCCGGATGCACCA
AGTGCGCGGCGGCACCGTTGATGCCAATCACACCACTGCCGCGTTCGCCGGTGATC
GCGTAGGTGACCAGTCGAGCACCGTTGTCGATATCGACGATGGTTACCTGTTCGCC
TTCCAGCAGGTCGGCGGCGTCCATCAAGTCGGCATCGATGGTCACCGAGCCGACGT
AGTGCAGGTCGGCGCAGGTCACCGTGGCGCGGTGGATCTTCGACTTCAGCATCGTC
CGTAACATCAGTTTCTCCAATGTGATTCGAGGATTGCCCGGTATCCGTCCGGGCGG
TCGGTGCCGGCGAAAGTTCCGATTTCAATCGCAATGTTGTCCAGCAGCCTGGTGGT
GCCAAGCCGGGCAGCAACCAGCAGCCGACCGGAACCGTTGAGCGGCATCGGGCCA
AGCCCGATATCGCGCAGCTCCAGGTAGTCGACCGCCACGCCGGGTGCAGCGTCGA
GCACCGCACGGGCGGCATCCAGCGCGGCCTGCGCGCCAGCCGTTGCCGCATGCGC
TGCGGCCGTTAGCGCCGCCGAGAGCGCGACGGCCGCCGCACGCTGGGCCGGGTCC
AGGTAGCGGTTGCGCGACGACATCGCCAGCCCGTCGGCTTCGCGCACGGTCGGCA
CGCCGACCACCGCGACATCGAGGTTGAAGTCCGCGACCAGCTGCCGGATCAGCAC
CAGCTGCTGGTAGTCCTTCTCACCGAAGAACACCCGATCCGGGCGCACGATCTGCA
GCAGCTTTAGCACGACCGTCAGCACGCCGGCGAAATGGGTTGGCCGCGGGCCGCC

CTCGAGTTCGGCGGCCAACGGACCGGGTTGCACGGTGGTGCGCAGGCCGTCGGGA
TACATCGCCGCGGTAGTTGGCGTGAAAGCGATTTCCACGCCTTCGGCCCGCAGTTG
CGCCAGGTCGTCGTCCGGGGTGCGGGATAGGCGTCGAGATCTTCCCCGGCACCG
AATTGCATCGGGTTGACGAAGATCGACACGACGACGACCGATCCGGGCACCCGCT
TGGCCGCACGCACCAACGCGAGGTGGCCTTCGTGCAGCGCACCCATAGTAGGCAC
CAACATCACTCGCCGGCCGGTGAGTCGCAGTGCGCGACTGACATCGGCGACATCCC
CCGGTGCCGAGTACACATTGA

SEQ ID NO:3 - A nadBC region of *Mycobacterium tuberculosis* H37Rv.

Deleted in Δ*nadBC* strain of Example 2.

AACGGGCGATGAGCCGGGACGCGTCGATGTACCGCGCCGCCGCCGGGCTGCACCG
GCTGTGCGACAGCCTATCCGGAGCACAGGTTCGCGACGTGGCTTGTCGCCGCGATT
TCGAGGACGTGGCGCTCACGCTGGTCGCGCAGAGCGTGACCGCCGCCGCCTTGGCC
CGCACCGAAAGCCGTGGCTGCCATCATCGCGCGGAGTACCCGTGCACCGTGCCGG
AGCAGGCACGCAGCATCGTGGTCCGGGGAGCCGACGACGCAAATGCGGTGTGTGT
CCAGGCGCTAGTGGCGGTGTGCTGATGGGGTTATCCGACTGGGAGCTGGCTGCGG
CTCGAGCAGCAATCGCGCGTGGGCTCGACGAGGACCTCCGGTACGGCCCGGATGT
CACCACATTGGCGACGGTGCCTGCCAGTGCGACGACCACCGCATCGCTGGTGACCC
GGGAGGCCGGTGTGGTTGCCGGATTGGATGTCGCGCTGCTGACGCTGAACGAAGT
CCTGGGCACCAACGGTTATCGGGTGCTCGACCGCGTCGAGGACGGCGCCCGGGTG
CCGCCGGGAGAGGCACTTATGACGCTGGAAGCCCAAACGCGCGGATTGTTGACCG
CCGAGCGCACCATGTTGAACCTGGTCGGTCACCTGTCGGGAATCGCCACCGCGACG
GCCGCGTGGGTCGATGCTGTGCGCGGGACCAAAGCGAAAATCCGCGATACCCGTA
AGACGCTGCCCGGCCTGCGCGCGCTGCAAAAATACGCGGTGCGTACCGGTGG

SEQ ID NO:4 - A *lysA* sequence deleted in Δ*lysA* strains

GTGAACGAGCTGCTGCACTTAGCGCCGAATGTGTGGCCGCGCAATACTACTCGCGA
TGAAGTCGGTGTGGTCTGCATCGCAGGAATTCCACTGACGCAGCTCGCCCAGGAGT
ACGGGACCCCGCTGTTCGTCATCGACGAGGACGACTTTCGCTCGCGCTGCCGAGAA
ACCGCCGCGGCCTTTGGAAGTGGGGCGAACGTGCACTATGCCGCCAAGGCGTTCCT
GTGCAGCGAAGTAGCCCGGTGGATCAGCGAAGAAGGGCTCTGTCTGGACGTTTGC
ACCGGTGGGGAGTTGGCGGTCGCGCTGCACGCTAGCTTTCCGCCCGAGCGAATTAC
CTTGCACGGCAACAACAAATCGGTCTCAGAGTTGACCGCTGCGGTCAAAGCCGGA
GTCGGCCATATTGTCGTCGATTCGATGACCGAGATCGAGCGCCTCGACGCCATCGC
GGGCGAGGCCGGAATCGTCCAGGATGTCCTGGTGCGTCTCACCGTCGGTGTCGAG
GCGCACACCCACGAGTTCATCTCCACCGCGCACGAGACGCGTCAGCCACATCGGTT
CGCAGATCTTCGACGTGGACGGCTTCGAACTCGCCGCGCACCGTGTCATCGGCCTG
CTACGCGACGTCGTCGGCGAGTTCGGTCCCGAAAAGACGGCACAGATCGCGACCG
TCGATCTCGGTGGCGGCTTGGGCATCTCGTATTTGCCGTCCGACGACCCACCGCCG
ATAGCCGAGCTCGCGGCCAAGCTGGGTACCATCGTGAGCGACGAGTCAACGGCCG
TGGGGCTGCCGACGCCCAAGCTCGTTGTGGAGCCCGGACGCGCCATCGCCGGACC
GGGCACCATCACGTTGTATGAGGTCGGCACCGTTAAGGACGTCGATGTCAGCGCC
ACAGCGCATCGACGTTACGTCAGTGTCGACGGCGGCATGAGCGACAACATCCGCA
CCGCGCTCTACGGCGCGCAGTATGACGTCCGGCTGGTGTCTCGAGTCAGCGACGCC
CCGCCGGTACCGGCCCGTCTGGTCGGAAAGCACTGCGAAAGTGGCGATATCATCG
TGCGGGACACCTGGGTGCCCGACGATATTCGGCCCGGCGATCTGGTTGCGGTTGCC

GCCACCGGCGCTTACTGCTATTCGCTGTCGAGTCGTTACAACATGGTCGGCCGTCC
CGCTGTGGTAGCGGTGCACGCGGGCAACGCTCGCCTGGTCCTGCGTCGGGAGACG
GTCGACGATTTGCTGAGTTTGGAAGTGAGGTGA

SEQ ID NO:5 - primer TH201

GGGGGCGCACCTCAAACC

SEQ ID NO:6 - primer TH202

ATGTGCCAATCGTCGACCAGAA

SEQ ID NO:7 - primer TH203

CACCCAGCCGCCCGGAT

SEQ ID NO:8 - primer TH204

TTCCTGATGCCGCCGTCTGA

SEQ ID NO:9 - primer Pan1

GTGCAGCGCCATCTCTCA

SEQ ID NO:10 - primer Pan2

GTTCACCGGGATGGAACG

SEQ ID NO:11 - primer Pan3

CCCGGCTCGGTGTGGGAT

SEQ ID NO:12 - primer Pan4

GCGCGGTATGCCCGGTAG